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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/52, 15/82, A01H 5/00	A1	(11) International Publication Number: WO 99/45122
		(43) International Publication Date: 10 September 1999 (10.09.99)

(21) International Application Number: PCT/US99/04999

(22) International Filing Date: 5 March 1999 (05.03.99)

(30) Priority Data:
60/077,107 6 March 1998 (06.03.98) US

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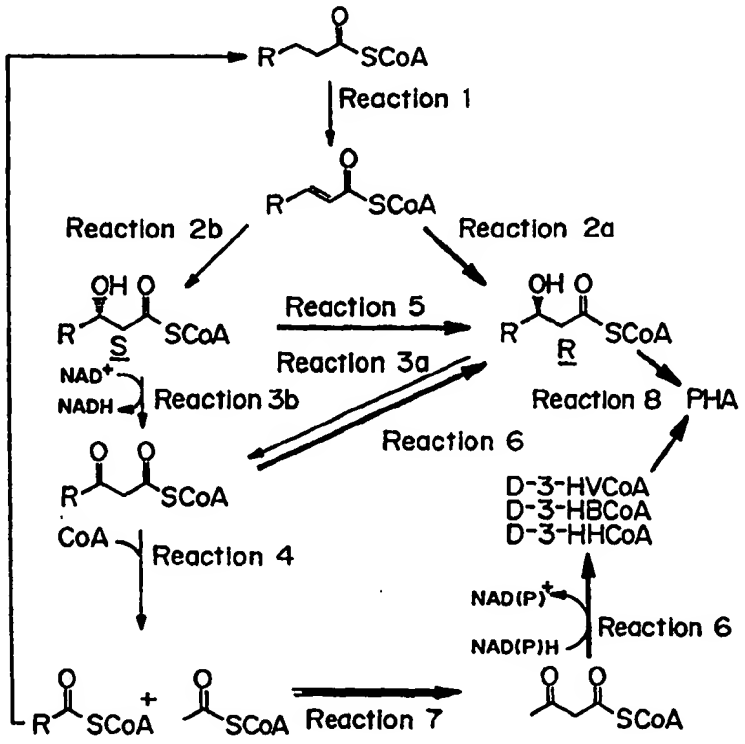
(81) Designated States: AU, CA, JP, MX, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MODIFICATION OF FATTY ACID METABOLISM IN PLANTS

(57) Abstract

Methods and systems to modify fatty acid biosynthesis and oxidation in plants to make new polymers are provided. Two enzymes are essential: a hydratase such as D–specific enoyl–CoA hydratase, for example, the hydratase obtained from *Aeromonas caviae*, and a β–oxidation enzyme system. Some plants have a β–oxidation enzyme system which is sufficient to modify polymer synthesis when the plants are engineered to express the hydratase. Examples demonstrate production of polymer by expression of these enzymes in transgenic plants. Examples also demonstrate that modifications in fatty acid biosynthesis can be used to alter plant phenotypes, decreasing or eliminating seed production and increasing green plant biomass, as well as producing polyhydroxyalkanoates.



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MODIFICATION OF FATTY ACID METABOLISM IN PLANTS

Background Of The Invention

The present invention is generally in the field of transgenic plant systems for the production of polyhydroxyalkanoate materials, modification of triglycerides and fatty acids, and methods for altering seed production in plants.

Methods for producing stable transgenic plants for agronomic crops have been developed over the last 15 years. Crops have been genetically modified for improvements in both input and output traits. In the former traits, tolerance to specific agrochemicals has been engineered into crops, and specific natural pesticides, such as the *Bacillus thuringenesis* toxin, have been expressed directly in the plant. There also has been significant progress in developing male sterility systems for the production of hybrid plants. With respect to output traits, crops are being modified to increase the value of the product, generally the seed, grain, or fiber of the plant. Critical metabolic targets include the modification of starch, fatty acid, and oil biosynthetic pathways.

There is considerable commercial interest in producing microbial polyhydroxyalkanoate (PHA) biopolymers in plant crops. See, for example, U.S. Patent Nos. 5,245,023 and 5,250,430 to Peoples and Sinskey; U.S. Patent No. 5,502,273 to Bright et al.; U.S. Patent No. 5,534,432 to Peoples and Sinskey; U.S. Patent No. 5,602,321 to John; U.S. Patent No. 5,610,041 to Somerville et al.; PCT WO 91/00917; PCT WO 92/19747; PCT WO 93/02187; PCT WO 93/02194; PCT WO 94/12014; Poirier et al., *Science* 256:520-23 (1992); van der Leij & Witholt, *Can. J. Microbiol.* 41(supplement):222-38 (1995); Nawrath & Poirier, *The International Symposium on Bacterial Polyhydroxyalkanoates*, (Eggink et al., eds.) Davos Switzerland (August 18-23, 1996); and Williams and Peoples, CHEMTECH 26: 38-44 (1996). PHAs are natural, thermoplastic polyesters and can be processed by traditional polymer techniques for use in an enormous variety

of applications, including consumer packaging, disposable diaper linings and garbage bags, food and medical products.

Early studies on the production of polyhydroxybutyrate in the chloroplasts of the experimental plant system *Arabidopsis thaliana* resulted in the accumulation of up to 14% of the leaf dry weight as PHB (Nawrath et al., 1993). *Arabidopsis*, however, has no agronomic value. Moreover, in order to economically produce PHAs in agronomic crops, it is desirable to produce the PHAs in the seeds, so that the current infrastructure for harvesting and processing seeds can be utilized. The options for recovery of the PHAs from plant seeds (PCT WO 97/15681) and the end use applications (Williams & Peoples, CHEMTECH 26:38-44 (1996)) are significantly affected by the polymer composition. Therefore, it would be advantageous to develop transgenic plant systems that produce PHA polymers having a well-defined composition.

Careful selection of the PHA biosynthetic enzymes on the basis of their substrate specificity allows for the production of PHA polymers of defined composition in transgenic systems (U.S. Patent Nos. 5,229,279; 5,245,023; 5,250,430; 5,480,794; 5,512,669; 5,534,432; 5,661,026; and 5,663,063).

In bacteria, each PHA group is produced by a specific pathway. In the case of the short pendant group PHAs, three enzymes are involved: β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase. The homopolymer PHB, for example, is produced by the condensation of two molecules of acetyl-coenzyme A to give acetoacetyl-coenzyme A. The latter then is reduced to the chiral intermediate R-3-hydroxybutyryl-coenzyme A by the reductase, and subsequently polymerized by the PHA synthase enzyme. The PHA synthase notably has a relatively wide substrate specificity which allows it to polymerize C3-C5 hydroxy acid monomers including both 4-hydroxy and 5-hydroxy acid units. This biosynthetic pathway is found in a number of bacteria such as *Alcaligenes eutrophus, A. latus, Azotobacter vinlandii,* and *Zoogloea ramigera*. Long pendant group PHAs are produced for example by many different *Pseudomonas* bacteria.

Their biosynthesis involve the β-oxidation of fatty acids and fatty acid synthesis as routes to the hydroxyacyl-coenzyme A monomeric units. The latter then are converted by PHA synthases which have substrate specificities favoring the larger C6-C14 monomeric units (Peoples & Sinskey, 1990).

In the case of the PHB-co-HX copolymers which usually are produced from cells grown on fatty acids, a combination of these routes can be responsible for the formation of the different monomeric units. Indeed, analysis of the DNA locus encoding the PHA synthase gene in *Aeromonas caviae*, which produces the copolymer PHB-co-3-hydroxyhexanoate, was used to identify a gene encoding a D-specific enoyl-CoA hydratase responsible for the production of the D-β-hydroxybutyryl-CoA and D-β - hydroxyhexanoyl-CoA units (Fukui & Doi, *J. Bacteriol.* 179:4821-30 (1997); Fukui et. al., *J. Bacteriol.* 180:667-73 (1998)). Other sources of such hydratase genes and enzymes include *Alcaligenes*, *Pseudomonas*, and *Rhodospirillum*.

The enzymes PHA synthase, acetoacetyl-CoA reductase, and β-ketothiolase, which produce the short pendant group PHAs in *A. eutrophus*, are coded by an operon comprising the *phb*C-*phb*A-*phb*B genes; Peoples *et al.*, 1987; Peoples & Sinskey, 1989). In the *Pseudomonas* organisms, the PHA synthases responsible for production of the long pendant group PHAs have been found to be encoded on the *pha* locus, specifically by the *pha*A and *pha*C genes (U.S. Patent Nos. 5,245,023 and 5,250,430; Huisman et. al., *J. Biol. Chem.* 266:2191-98 (1991)). Since these earlier studies, a range of PHA biosynthetic genes have been isolated and characterized or identified from genome sequencing projects. Examples of known PHA biosynthetic genes are disclosed in the following references: *Aeronomas caviae* (Fukui & Doi, 1997, *J. Bacteriol.* 179:4821-30); *Alcaligenes eutrophus* (U.S. Patent Nos. 5,245,023; 5,250,430; 5,512,669; and 5,661,026; Peoples & Sinskey, *J. Biol. Chem.* 264:15298-03 (1989)); *Acinetobacter* (Schembri et. al., *FEMS Microbiol. Lett.* 118:145-52 (1994)); *Chromatium vinosum* (Liebergesell & Steinbuchel, *Eur. J. Biochem.* 209:135-50 (1992)); *Methylobacterium extorquens* (Valentin & Steinbuchel, *Appl. Microbiol. Biotechnol.* 39:309-17

(1993)); *Nocardia corallina* (GENBANK Accession No. AF019964; Hall et. al., 1998, *Can. J. Microbiol.* 44:687-69); *Paracoccus denitrificans* (Ueda et al., *J. Bacteriol.* 178:774-79 (1996); Yabutani et. al., *FEMS Microbiol. Lett.* 133:85-90 (1995)); *Pseudomonas acidophila* (Umeda et. al., 1998, *Applied Biochemistry and Biotechnology*, 70-72:341-52); *Pseudomonas sp.* 61-3 (Matsusaki et al., 1998, *J. Bacteriol.* 180:6459-67); *Nocardia corallina*; *Pseudomonas aeruginosa* (Timm & Steinbuchel, *Eur. J. Biochem.* 209:15-30 (1992)); *P. oleovorans* (U.S. Patent Nos. 5,245,023 and 5,250,430; Huisman et. al., *J. Biol. Chem.* 266(4):2191-98 (1991); *Rhizobium etli* (Cevallos et. al., *J. Bacteriol.* 178:1646-54 (1996)); *R. meliloti* (Tombolini et. al., *Microbiology* 141:2553-59 (1995)); *Rhodococcus ruber* (Pieper-Furst & Steinbuchel, FEMS Microbiol. Lett. 75:73-79 (1992)); *Rhodospirillum rubrum* (Hustede et. al., *FEMS Microbiol. Lett* 93:285-90 (1992)); *Rhodobacter sphaeroides* (Hustede et. al., *FEMS Microbiol. Rev.* 9:217-30 (1992); *Biotechnol. Lett.* 15:709-14 (1993); *Synechocystis sp.* (*DNA Res.* 3:109-36 (1996)); *Thiocapsiae violacea* (*Appl. Microbiol. Biotechnol.* 38:493-501 (1993)) and *Zoogloea ramigera* (Peoples et. al., *J. Biol. Chem.* 262:97-102 (1987); Peoples & Sinskey, *Molecular Microbiology* 3:349-57 (1989)). The availability of these genes or their published DNA sequences should provide a range of options for producing PHAs.

PHA synthases suitable for producing PHB-co-HH copolymers comprising from 1-99% HH monomers are encoded by the *Rhodococcus ruber*, *Rhodospirillum rubrum*, *Thiocapsiae violacea*, and *Aeromonas caviae* PHA synthase genes. PHA synthases useful for incorporating 3-hydroxyacids of 6-12 carbon atoms in addition to R-3-hydroxybutyrate i.e. for producing biological polymers equivalent to the chemically synthesized copolymers described in PCT WO 95/20614, PCT WO 95/20615, and PCT WO 95/20621 have been identified in a number of *Pseudomonas* and other bacteria (Steinbüchel & Wiese, *Appl. Microbiol. Biotechnol.* 37:691-97 (1992); Valentin et al., *Appl. Microbiol. Biotechnol.* 36:507-14 (1992); Valentin et al., *Appl. Microbiol. Biotechnol.* 40:710-16 (1994); Lee et al., *Appl. Microbiol. Biotechnol.* 42:901-09 (1995); Kato et al., *Appl. Microbiol.*

Biotechnol. 45:363-70 (1996); Abe et al., *Int. J. Biol. Macromol.* 16:115-19 (1994); Valentin et al., *Appl. Microbiol. Biotechnol.* 46:261-67 (1996)) and can readily be isolated as described in U.S. Patent Nos. 5,245,023 and 5,250,430. The PHA synthase from *P. oleovorans* (U.S. Patent Nos. 5,245,023 and 5,250,430; Huisman et. al., *J. Biol. Chem.* 266(4): 2191-98 (1991)) is suitable for producing the long pendant group PHAs. Plant genes encoding β-ketothiolase also have been identified (Vollack & Bach, *Plant Physiol.* 111:1097-107 (1996)).

Despite this ability to modify monomer composition by selection of the syntheses and substrates, it is desirable to modify other features of polymer biosynthesis, such as that which involves fatty acid metabolism.

It is therefore an object of the present invention to provide a method and DNA constructs to introduce fatty acid oxidation enzyme systems for manipulating the cellular metabolism of plants.

It is another object of the present invention to provide methods for enhancing the production of PHAs in plants, preferably in the oilseeds thereof.

Summary Of The Invention

Methods and systems to modify fatty acid biosynthesis and oxidation in plants to make new polymers are described. Two enzymes are essential: a hydratase such as D-specific enoyl-CoA hydratase, for example, the hydratase obtained from *Aeromonas caviae*, and a β-oxidation enzyme system. Some plants have a β-oxidation enzyme system which is sufficient to modify polymer synthesis when the plants are engineered to express the hydratase.

Examples demonstrate production of polymer by expression of these enzymes in transgenic plants. Examples also demonstrate that modifications in fatty acid biosynthesis can be used to alter plant phenotypes, decreasing or eliminating seed production and increasing green plant biomass, as well as producing PHAs.

Brief Description of the Drawings

Figure 1 is a schematic of fatty acid β-oxidation routes to produce polyhydroxyalkanoate monomers.

Figure 2 is a schematic showing plasmid constructs pSBS2024 and pSBS2025.

Figures 3A and 3B are schematics showing plasmid constructs pCGmf124 and pCGmf125.

Figures 4A and 4B are schematics showing plasmid constructs pmf1249 and pmf1254.

Figures 5A and 5B are schematics showing plasmid constructs pCGmf224 and pCGmf225.

Figures 6A and 6B are schematics showing plasmid constructs pCGmf1P2S and pCGmf2P1S.

Detailed Description Of The Invention

Methods and DNA constructs for manipulating the cellular metabolism of plants by introducing fatty acid oxidation enzyme systems into the cytoplasm or plastids of developing oilseeds or green tissue are provided. Fatty acid oxidation systems typically comprise several enzyme activities including a β-ketothiolase enzyme activity which utilizes a broad range of β-ketoacyl-CoA substrates.

It surprisingly was found that expression of at least one of these transgenes from the bean phaseolin promoter results in male sterility. Interestingly, these plants did not set seed, but instead produced higher than normal levels of biomass (e.g., leafs, stems, stalks). Therefore the methods and constructs described herein also can be used to create male sterile plants, for example, for hybrid production or to increase the production of biomass of forage, such as alfalfa or tobacco. Plants generated using these methods and DNA constructs are useful for producing polyhydroxyalkanoate biopolymers or for producing novel oil compositions.

The methods described herein include the subsequent incorporation of additional transgenes, in particular encoding additional enzymes involved

in fatty acid oxidation or polyhydroxyalkanoate biosynthesis. For polyhydroxyalkanoate biosynthesis, the methods include the incorporation of transgenes encoding enzymes, such as NADH and/or NADPH acetoacetyl-CoenzymeA reductases, PHB synthases, PHA synthases, acetoacetyl-CoA thiolase, hydroxyacyl-CoA epimerases, delta3-cis-delta2-trans enoyl-CoA isomerases, acyl-CoA dehydrogenase, acyl-CoA oxidase and enoyl-CoA hydratases by subsequent transformation of the transgenic plants produced using the methods and DNA constructs described herein or by traditional plant breeding methods.

I. Plant Expression Systems

In a preferred embodiment, the fatty acid oxidation transgenes are expressed from a seed specific promoter, and the proteins are expressed in the cytoplasm of the developing oilseed. In an alternate preferred embodiment, fatty acid oxidation transgenes are expressed from a seed specific promoter and the expressed proteins are directed to the plastids using plastid targeting signals. In another preferred embodiment, the fatty acid oxidation transgenes are expressed directly from the plastid chromosome where they have been integrated by homologous recombination. The fatty acid oxidation transgenes may also be expressed throughout the entire plant tissue from a constitutive promoter. It is also useful to be able to control the expression of these transgenes by using promoters that can be activated following the application of an agrochemical or other active ingredient to the crop in the field. Additional control of the expression of these genes encompassed by the methods described herein include the use of recombinase technologies for targeted insertion of the transgenes into specific chromosomal sites in the plant chromosome or to regulate the expression of the transgenes.

The methods described herein involve a plant seed having a genome including (a) a promoter operably linked to a first DNA sequence and a 3'-untranslated region, wherein the first DNA sequence encodes a fatty acid oxidation polypeptide and optionally (b) a promoter operably linked to a second DNA sequence and a 3'-untranslated region, wherein the second

DNA sequence encodes a fatty acid oxidation polypeptide. Expression of the two transgenes provides the plant with a functional fatty acid ß-oxidation system having at least β-ketothiolase, dehydrogenase and hydratase activities in the cytoplasm or plastids other than peroxisomes or glyoxisomes. The first and/or second DNA sequence may be isolated from bacteria, yeast, fungi, algae, plants, or animals. It is preferable that at least one of the DNA sequences encodes a polypeptide with at least two, and preferably three, enzyme activities.

Transformation Vectors

DNA constructs useful in the methods described herein include transformation vectors capable of introducing transgenes into plants. Several plant transformation vector options are available, including those described in "Gene Transfer to Plants" (Potrykus, et al., eds.) Springer-Verlag Berlin Heidelberg New York (1995); "Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins" (Owen, et al., eds.) John Wiley & Sons Ltd. England (1996); and "Methods in Plant Molecular Biology: A Laboratory Course Manual" (Maliga, et al. eds.) Cold Spring Laboratory Press, New York (1995). Plant transformation vectors generally include one or more coding sequences of interest under the transcriptional control of 5' and 3' regulatory sequences, including a promoter, a transcription termination and/or polyadenylation signal, and a selectable or screenable marker gene. The usual requirements for 5' regulatory sequences include a promoter, a transcription termination and/or a polyadenylation signal. For the expression of two or more polypeptides from a single transcript, additional RNA processing signals and ribozyme sequences can be engineered into the construct (U.S. Patent No. 5,519,164). This approach has the advantage of locating multiple transgenes in a single locus, which is advantageous in subsequent plant breeding efforts. An additional approach is to use a vector to specifically transform the plant plastid chromosome by homologous recombination (U.S. Patent No. 5,545,818), in which case it is possible to take advantage of the prokaryotic nature of the plastid genome and insert a number of transgenes as an operon.

Promoters

A large number of plant promoters are known and result in either constitutive, or environmentally or developmentally regulated expression of the gene of interest. Plant promoters can be selected to control the expression of the transgene in different plant tissues or organelles for all of which methods are known to those skilled in the art (Gasser & Fraley, *Science* 244:1293-99 (1989)). The 5' end of the transgene may be engineered to include sequences encoding plastid or other subcellular organelle targeting peptides linked in-frame with the transgene. Suitable constitutive plant promoters include the cauliflower mosaic virus 35S promoter (CaMV) and enhanced CaMV promoters (Odell et. al., *Nature*, 313: 810 (1985)), actin promoter (McElroy et al., *Plant Cell* 2:163-71 (1990)), AdhI promoter (Fromm et. al., *Bio/Technology* 8:833-39 (1990); Kyozuka et al., *Mol. Gen. Genet.* 228:40-48 (1991)), ubiquitin promoters, the Figwort mosaic virus promoter, mannopine synthase promoter, nopaline synthase promoter and octopine synthase promoter. Useful regulatable promoter systems include spinach nitrate-inducible promoter, heat shock promoters, small subunit of ribulose biphosphate carboxylase promoters and chemically inducible promoters (U.S. Patent No. 5,364,780 to Hershey et al.).

In a preferred embodiment of the methods described herein, the transgenes are expressed only in the developing seeds. Promoters suitable for this purpose include the napin gene promoter (U.S. Patent Nos. 5,420,034 and 5,608,152), the acetyl-CoA carboxylase promoter (U.S. Patent Nos. 5,420,034 and 5,608,152), 2S albumin promoter, seed storage protein promoter, phaseolin promoter (Slightom et. al., *Proc. Natl. Acad. Sci. USA* 80:1897-1901 (1983)), oleosin promoter (Plant et. al., *Plant Mol. Biol.* 25:193-205 (1994); Rowley et al., *Biochim. Biophys. Acta.* 1345:1-4 (1997); U.S. Patent No. 5,650,554; and PCT WO 93/20216), zein promoter, glutelin promoter, starch synthase promoter, and starch branching enzyme promoter.

The transformation of suitable agronomic plant hosts using these vectors can be accomplished with a variety of methods and plant tissues.

Representative plants useful in the methods disclosed herein include the *Brassica* family including *napus, rappa, sp. carinata* and *juncea*; maize; soybean; cottonseed; sunflower; palm; coconut; safflower; peanut; mustards including *Sinapis alba*; and flax. Crops harvested as biomass, such as silage corn, alfalfa, or tobacco, also are useful with the methods disclosed herein. Representative tissues for transformation using these vectors include protoplasts, cells, callus tissue, leaf discs, pollen, and meristems. Representative transformation procedures include *Agrobacterium*-mediated transformation, biolistics, microinjection, electroporation, polyethylene glycol-mediated protoplast transformation, liposome-mediated transformation, and silicon fiber-mediated transformation (U.S. Patent No. 5,464,765; "Gene Transfer to Plants" (Potrykus, et al., eds.) Springer-Verlag Berlin Heidelberg New York (1995); "Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins" (Owen, et al., eds.) John Wiley & Sons Ltd. England (1996); and "Methods in Plant Molecular Biology: A Laboratory Course Manual" (Maliga, et al. eds.) Cold Spring Laboratory Press, New York (1995)).

II. Methods for Making and Screening for Transgenic Plants

In order to generate transgenic plants using the constructs described herein, the following procedures can be used to obtain a transformed plant expressing the transgenes subsequent to transformation: select the plant cells that have been transformed on a selective medium; regenerate the plant cells that have been transformed to produce differentiated plants; select transformed plants expressing the transgene at such that the level of desired polypeptide is obtained in the desired tissue and cellular location.

For the specific crops useful for practicing the described methods, transformation procedures have been established, as described for example, in "Gene Transfer to Plants" (Potrykus, et al., eds.) Springer-Verlag Berlin Heidelberg New York (1995); "Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins" (Owen, et al., eds.) John Wiley & Sons Ltd. England (1996); and "Methods in Plant Molecular Biology: A Laboratory Course Manual" (Maliga, et al. eds.) Cold Spring Laboratory

Press, New York (1995).

Brassica napus can be transformed as described, for example, in U.S. Patent Nos. 5,188,958 and 5,463,174. Other *Brassica* such as *rappa, carinata* and *juncea* as well as *Sinapis alba* can be transformed as described by Moloney et. al., *Plant Cell Reports* 8:238-42 (1989). Soybean can be transformed by a number of reported procedures (U.S. Patent Nos. 5,015,580; 5,015,944; 5,024,944; 5,322,783; 5,416,011; and 5,169,770). Several transformation procedures have been reported for the production of transgenic maize plants including pollen transformation (U.S. Patent No. 5,629,183), silicon fiber-mediated transformation (U.S. Patent No. 5,464,765), electroporation of protoplasts (U.S. Patent Nos. 5,231,019; 5,472,869; and 5,384,253) gene gun (U.S. Patent Nos. 5,538,877 and 5,538,880 and *Agrobacterium*-mediated transformation (EP 0 604 662 A1; PCT WO 94/00977). The *Agrobacterium*-mediated procedure is particularly preferred, since single integration events of the transgene constructs are more readily obtained using this procedure, which greatly facilitates subsequent plant breeding. Cotton can be transformed by particle bombardment (U.S. Patent Nos. 5,004,863 and 5,159,135). Sunflower can be transformed using a combination of particle bombardment and *Agrobacterium* infection (EP 0 486 233 A2; U.S. Patent No. 5,030,572). Flax can be transformed by either particle bombardment or *Agrobacterium*-mediated transformation. Recombinase technologies include the *cre-lox*, FLP/FRT, and Gin systems. Methods for utilizing these technologies are described for example in U.S. Patent No. 5,527,695 to Hodges et al.; Dale & Ow, *Proc. Natl. Acad. Sci. USA* 88:10558-62 (1991); Medberry et. al., *Nucleic Acids Res.* 23:485-90 (1995).

Selectable Marker Genes

Selectable marker genes useful in practicing the methods described herein include the neomycin phosphotransferase gene nptII (U.S. Patent Nos. 5,034,322 and 5,530,196), hygromycin resistance gene (U.S. Patent No. 5,668,298), bar gene encoding resistance to phosphinothricin (U.S. Patent No. 5,276,268). EP 0 530 129 A1 describes a positive selection system

which enables the transformed plants to outgrow the non-transformed lines by expressing a transgene encoding an enzyme that activates an inactive compound added to the growth media. Screenable marker genes useful in the methods herein include the β-glucuronidase gene (Jefferson et. al., *EMBO J.* 6:3901-07 (1987); U.S. Patent No. 5,268,463) and native or modified green fluorescent protein gene (Cubitt et. al., *Trends Biochem Sci.* 20:448-55 (1995); Pang et. al., *Plant Physiol.* 112:893-900 (1996)). Some of these markers have the added advantage of introducing a trait, such as herbicide resistance, into the plant of interest, thereby providing an additional agronomic value on the input side.

In a preferred embodiment of the methods described herein, more than one gene product is expressed in the plant. This expression can be achieved via a number of different methods, including (1) introducing the encoding DNAs in a single transformation event where all necessary DNAs are on a single vector; (2) introducing the encoding DNAs in a co-transformation event where all necessary DNAs are on separate vectors but introduced into plant cells simultaneously; (3) introducing the encoding DNAs by independent transformation events successively into the plant cells i.e. transformation of transgenic plant cells expressing one or more of the encoding DNAs with additional DNA constructs; and (4) transformation of each of the required DNA constructs by separate transformation events, obtaining transgenic plants expressing the individual proteins and using traditional plant breeding methods to incorporate the entire pathway into a single plant.

III. β-Oxidation Enzyme Pathways

Production of PHAs in the cytosol of plants requires the cytosolic localization of enzymes that are able to produce R-3-hydroxyacyl CoA thioesters as substrates for PHA synthases. Both eukaryotes and prokaryotes possess a β-oxidation pathway for fatty acid degradation that consists of a series of enzymes that convert fatty acyl CoA thioesters to acetyl CoA. While these pathways proceed via intermediate 3-hydroxyacyl CoA, the stereochemistry of this intermediate varies among organisms. For example,

the β-oxidation pathways of bacteria and the peroxisomal pathway of higher eukaryotes degrade fatty acids to acetyl CoA via S-3-hydroxyacyl CoA (Schultz, "Oxidation of Fatty Acids" in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance et al., eds.) pp. 101-06 (Elsevier, Amsterdam 1991)). In *Escherichia coli*, an epimerase activity encoded by the β-oxidation multifunctional enzyme complex is capable of converting S-3-hydroxyacyl CoA to R-3-hydroxyacyl CoA. Yeast possesses a peroxisomal localized fatty acid degradation pathway that proceeds via intermediate R-3-hydroxyacyl CoA (Hiltunen, et al. *J. Biol. Chem.* 267: 6646-53 (1992); Filppula, et al. *J. Biol. Chem.* 270:27453-57 (1995)), such that no epimerase activity is required to produce PHAs.

Plants, like other higher eukaryotes, possesses a β-oxidation pathway for fatty acid degradation localized subcellularly in the peroxisomes (Gerhardt, "Catabolism of Fatty Acids [α and β Oxidation]" in *Lipid Metabolism in Plants* (Moore, Jr., ed.) pp. 527-65 (CRC Press, Boca Raton, Florida 1993)). Production of PHAs in the cytosol of plants therefore necessitates the cytosolic expression of a β-oxidation pathway, for conversion of fatty acids to R-3-hydroxyacyl CoA thioesters of the correct chain length, as well as cytosolic expression of an appropriate PHA synthase, to polymerize R-3-hydroxyacyl CoA to polymer.

Fatty acids are synthesized as saturated acyl-ACP thioesters in the plastids of plants (Hartwood, "Plant Lipid Metabolism" in *Plant Biochemistry* (Dey et al., eds.) pp. 237-72 (Academic Press, San Diego 1997)). Prior to export from the plastid into the cytosol, the majority of fatty acids are desaturated via a Δ9 desaturase. The pool of newly synthesized fatty acids in most oilseed crops consists predominantly of oleic acid (*cis* 9-octadecenoic acid), stearic acid (octadecanoic acid), and palmitic acid (hexadecanoic acid). However, some plants, such as coconut and palm kernel, synthesize shorter chain fatty acids (C8-14). The fatty acid is released from ACP via a thioesterase and subsequently converted to an acyl-CoA thioester via an acyl CoA synthetase located in the plastid membrane (Andrews, et al., "Fatty acid and lipid biosynthesis and degradation" in *Plant*

Physiology, Biochemistry, and Molecular Biology (Dennis et al., eds.) pp. 345-46 (Longman Scientific & Technical, Essex, England 1990); Harwood, "Plant Lipid Metabolism" in *Plant Biochemistry* (Dey et al., eds) p. 246 (Academic Press, San Diego 1997)).

The cytosolic conversion of the pool of newly synthesized acyl CoA thioesters via fatty acid degradation pathways and the conversion of intermediates from these series of reactions to R-3-hydroxyacyl-CoA substrates for PHA synthases can be achieved via the enzyme reactions outlined in Figure 1. The PHA synthase substrates are C4-C16 R-3-hydroxyacyl CoAs. For saturated fatty acyl CoAs, conversion to R-3-hydroxyacyl CoA thioesters using fatty acids degradation pathways necessitates the following sequence of reactions: conversion of the acyl CoA thioester to *trans*-2-enoyl-CoA (reaction 1), hydration of *trans*-2-enoyl-CoA to R-3-hyddroxy acyl CoA (reaction 2a, e.g. yeast system operates through this route and the *Aeromonas caviae* D-specific hydratase yields C4-C7 R-3-hydroxyacyl-CoAs), hydration of *trans*-2-enoyl-CoA to S-3-hydroxy acyl CoA (reaction 2b), and epimerization of S-3-hydroxyacyl CoA to R-3-hydroxyacyl CoA (reaction 5, e.g. cucumber tetrafunctional protein, bacterial systems). If 3-hydroxyacyl CoA is not polymerized by PHA synthase forming PHA, it can proceed through the remainder of the β-oxidation pathway as follows: oxidation of 3-hydroxyacyl CoA to form β-keto acyl CoA (reaction 3) followed by thiolysis in the presence of CoA to yield acetyl CoA and a saturated acyl CoA thioester shorter by two carbon units (reaction 4). The acyl CoA thioester produced in reaction 4 is free to re-enter the β-oxidation pathway at reaction 1 and the acetyl-CoA produced can be converted to R-3-hydroxyacyl CoA by the action of β-ketothiolase (reaction 7) and NADH or NADPH acetoacetyl-CoA reductase (reaction 6). This latter route is useful for producing R-3-hydroxybutyryl-CoA, R-3-hydroxyvaleryl-CoA and R-3-hydroxyhexanoyl-CoA. The R-3-hydroxyacids of four to sixteen carbon atoms produced by this series of enzymatic reactions can be polymerized by PHA synthases expressed from a transgene, or transgenes in the case of the two subunit synthase enzymes,

into PHA polymers.

For Δ9 unsaturated fatty acyl CoAs, a variation of the reaction sequences described is required. Three cycles of β-oxidation, as detailed in Figure 1, will remove six carbon units yielding an unsaturated acyl CoA thioester with a *cis* double bond at position 3. Conversion of the *cis* double bond at position 3 to a *trans* double bond at position 2, catalyzed by Δ^3-*cis*-Δ^2-*trans*-enoyl CoA isomerase will allow the β-oxidation reaction sequences outlined in Figure 1 to proceed. This enzyme activity is present on the microbial β-oxidation complexes and the plant tetrafunctional protein, but not on the yeast *fox*1.

Acyl CoA thioesters also can be degraded to a β-keto acyl CoA and converted to R-3-hydroxyacyl CoA via a NADH or NADPH dependent reductase (reaction 6).

Multifunctional enzymes that encode S-specific hydratase, S-specific dehydrogenase, β-ketothiolase, epimerase and Δ^3-*cis*-Δ^2-*trans*-enoyl CoA isomerase activities have been found in bacteria such as *Escherichia coli* (Spratt, et al., *J. Bacteriol.* 158:535-42 (1984)) and *Pseudomonas fragi* (Immure, et al., *J. Biochem.* 107:184-89 (1990)). The multifunctional enzyme complexes consist of two copies of each of two subunits such that catalytically active protein forms a heterotetramer. The hydratase, dehydrogenase, epimerase, and Δ^3-*cis*-Δ^2-*trans*-enoyl CoA isomerase activities are located on one subunit, whereas the thiolase is located on another subunit. The genes encoding the enzymes from organisms such as *E. coli* (Spratt, et al., *J. Bacteriol.* 158:535-42 (1984); DiRusso, *J. Bacteriol.* 172:6459-68 (1990)) and *P. fragi* (Sato, et al., *J. Biochem.* 111:8-15 (1992)) have been isolated and sequenced and are suitable for practicing the methods described herein. Furthermore, the *E. coli* enzyme system has been subjected to site-directed mutagenesis analysis to identify amino acid residues critical to the individual enzyme activities (He & Yang, *Biochemistry* 35:9625-30 (1996); Yang et. al., *Biochemistry* 34:6641-47 (1995); He & Yang, *Biochemistry* 36:11044-49 (1997); He et. al., *Biochemistry* 36:261-68 (1997); Yang & Elzinga, *J. Biol. Chem.* 268:6588-

92 (1993)). These mutant genes also could be used in some embodiments of the methods described herein.

Mammals, such as rat, possess a trifunctional β-oxidation enzyme in their peroxisomes that contains hydratase, dehydrogenase, and Δ^3-*cis*-Δ^2-*trans*-enoyl CoA isomerase activities. The trifunctional enzyme from rat liver has been isolated and has been found to be monomeric with a molecular weight of 78 kDa (Palosaari, et al., *J. Biol. Chem.* 265:2446-49 (1990)). Unlike the bacterial system, thiolase activity is not part of the multienzyme protein (Schultz, "Oxidation of Fatty Acids" in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance et al., eds) p. 95 (Elsevier, Amsterdam (1991)). Epimerization in rat occurs by the combined activities of two distinct hydratases, one which converts R-3-hydroxyacyl CoA to *trans*-2-enoyl CoA, and another which converts *trans*-2-enoyl CoA to S-3-hydroxyacyl CoA (Smeland, et al., *Biochemical and Biophysical Research Communications* 160:988-92 (1989)). Mammals also possess β-oxidation pathways in their mitochondria that degrade fatty acids to acetyl CoA via intermediate S-3-hydroxyacyl CoA (Schultz, "Oxidation of Fatty Acids" in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance et al., eds) p. 96 (Elsevier, Amsterdam (1991)). Genes encoding mitochondrial β-oxidation activities have been isolated from several animals including a Rat mitochondrial long chain acyl CoA hydratase/3-hydroxy acyl CoA dehydrogenase (GENBANK Accession # D16478) and a Rat mitochondrial thiolase (GENBANK Accession #s D13921, D00511).

Yeast possesses a multifunctional enzyme, Fox2, that differs from the β-oxidation complexes of bacteria and higher eukaryotes in that it proceeds via a R-3-hydroxyacyl CoA intermediate instead of S-3-hydroxyacyl CoA (Hiltunen, et al., *J. Biol. Chem.* 267:6646-53 (1992)). Fox2 possesses R-specific hydratase and R-specific dehydrogenase enzyme activities. This enzyme does not possess the Δ^3-*cis*-Δ^2-*trans*-enoyl CoA isomerase activity needed for degradation of Δ9-*cis*-hydroxyacyl CoAs to form R-3-hydroxyacyl CoAs. The gene encoding *fox2* from yeast has been isolated and sequenced and encodes a 900 amino acid protein. The DNA sequence of

the structural gene and amino acid sequence of the encoded polypeptide is shown in SEQ ID NO:1 and SEQ ID NO:2.

Plants have a tetrafunctional protein similar to the yeast Fox2, but also encoding a Δ^3-*cis*-Δ^2-*trans*-enoyl CoA isomerase activity (Muller et., al., *J. Biol. Chem.* 269:20475-81 (1994)). The DNA sequence of the cDNA and amino acid sequence of the encoded polypeptide is shown in SEQ ID NO:3 and SEQ ID NO:4.

IV. Targeting of Enzymes to the Cytoplasm of Oil Seed Crops

Engineering PHA production in the cytoplasm of plants requires directing the expression of β-oxidation to the cytosol of the plant. No targeting signals are present in the bacterial systems, such as faoAB. In fungi, yeast, plants, and mammals, β-oxidation occurs in subcellular organelles. Typically, the genes are expressed from the nuclear chromosome, and the polypeptides synthesized in the cytoplasm are directed to these organelles by the presence of specific amino acid sequences. To practice the methods described herein using genes isolated from eukaryotic sources, e.g., fatty acid oxidation enzymes from eukaryotic sources, such as yeast, fungi, plants, and mammals, the removal or modification of subcellular targeting signals is required to direct the enzymes to the cytosol. It may be useful to add signals for directing proteins to the endoplasmic reticulum. Peptides useful in this process are well known in the art. The general approach is to modify the transgene by inserting a DNA sequence specifying an ER targeting peptide sequence to form a chimeric gene.

Eukaryotic acyl CoA dehydrogenases, as well as other mitrochondrial proteins, are targeted to the mitochondria via leader peptides on the N-terminus of the protein that are usually 20-60 amino acids long (Horwich, *Current Opinion in Cell Biology*, 2:625-33 (1990)). Despite the lack of an obvious consensus sequence for mitochondrial import leader peptides, mutagenesis of key residues in the leader sequence have been demonstrated to prevent the import of the mitochrondrial protein. For example, the import of *Saccharomyces cerevisiae* F1-ATPase was prevented by mutagenesis of its leader sequence, resulting in the accumulation of the modified precursor

protein in the cytoplasm (Bedwell, et al., *Mol. Cell Biol.* 9:1014-25 (1989))

Three eukaryotic peroxisomal targeting signals have been reported (Gould, et al., *J. Cell Biol.* 108:1657-64 (1989); Brickner, et al., *J. Plant Physiol.*, 113:1213-21 (1997)). The tripeptide targeting signal S/A/C-K/H/R-L occurs at the C-terminal end of many peroxisomal proteins (Gould, et al., *J. Cell Biol.* 108:1657-64 (1989)). Mutagenesis of this sequence has been shown to prevent import of proteins into peroxisomes. Some peroxisomal proteins do not contain the tripeptide at the C-terminal end of the protein. For these proteins, it has been suggested that targeting occurs via the tripeptide in an internal position within the protein sequence (Gould, et al., *J. Cell Biol.* 108:1657-64 (1989)) or via an unknown, unrelated sequence (Brickner, et al., *J. Plant Physiol.* 113:1213-21 (1997)). The results of *in vitro* peroxisomal targeting experiments with fragments of acyl CoA oxidase from *Candida tropicalis* appear to support the latter theory and suggest that there are two separate targeting signals within the internal amino acid sequence of the polypeptide (Small, et al., *The EMBO Journal* 7:1167-73 (1988)). In the aforementioned study, the targeting signals were localized to two regions of 118 amino acids in length, and neither of regions was found to contain the targeting signal S/A/C-K/H/R-L. A small number of peroxisomal proteins appear to contain an amino terminal leader sequence for import into peroxisomes (Brickner, et al., *J. Plant Physiol.* 113:1213-21 (1997)). These targeting signals can be deleted or altered by site directed mutagenesis.

V. Cultivation and Harvesting of Transgenic Plant

The transgenic plants can be grown using standard cultivation techniques. The plant or plant part also can be harvested using standard equipment and methods. The PHAs can be recovered from the plant or plant part using known techniques such as solvent extraction in conjunction with traditional seed processing technologies, as described in PCT WO 97/15681, or can be used directly, for example, as animal feed, where it is unnecessary to extract the PHA from the plant biomass.

Several lines which did not produce seed, produced much higher

levels of biomass. produced much higher levels of biomass. This phenotype therefore may be useful as a means to increase the amount of green biomass produced per acre for silage, forage, or other biomass crops. End uses include the more cost effective production of forage crops for animal feed or as energy crops for electric power generation. Other uses include increasing biomass levels in crops, such as alfalfa or tobacco, for subsequent recovery of industrial products, such as PHAs by extraction.

The compositions and methods of preparation and use thereof described herein are further described by the following non-limiting examples.

Example 1: Isolation and Characterization of the *Pseudomonas putida faoAB* Genes and Fao Enzyme

All DNA manipulations, including PCR, DNA sequencing *E. coli* transformation, and plasmids purification, were performed using standard procedures, as described, for example, by Sambrook et. al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, New York (1989)). The genes encoding *faoAB* from *Pseudomonas putida* were isolated using a probe generated from *P. putida* genomic DNA by PCR (polymerase chain reaction) using primers 1 and 2 possessing homology to *faoB* from *Pseudomonas fragi* (Sato, et al., *J. Biochem.* 111:8-15 (1992)).

Primer 1:

5' gat ggg ccg ctc caa ggg tgg 3' (SEQ ID NO:5)

Primer 2:

5' caa ccc gaa ggt gcc gcc att 3' (SEQ ID NO:6)

A 1.1 kb DNA fragment was purified from the PCR reaction and used as a probe to screen a *P. putida* genomic library constructed in plasmid pBKCMV using the lambda ZAP expression system (Stratagene). Plasmid pMFX1 was selected from the positive clones and the DNA sequence of the insert containing the *fao*AB genes and flanking sequences determined. This is shown in SEQ ID NO:7. A fragment containing *faoAB* was subcloned with the native *P. putida* ribosome binding site intact into the expression

vector pTRCN forming plasmid pMFX3 as follows. Plasmid pMFX1 was digested with *Bsr*G I. The resulting protruding ends were filled in with Klenow. Digestion with *Hind* III yielded a 3.39 kb blunt ended/*Hind* III fragment encoding FaoAB. The expression vector pTRCN was digested with *Sma* I/*Hind* III and ligated with the *faoAB* fragment forming the 7.57 kb plasmid pMFX3.

Enzymes in the FaoAB multienzyme complex were assayed as follows. Hydratase activity was assayed by monitoring the conversion of NAD to NADH using the coupling enzyme L-β-hydroxyacyl CoA dehydrogenase as previously described, except that assays were run in the presence of CoA (Filppula, et al., *J. Biol. Chem.* 270:27453-57 (1995)). Severe product inhibitation of the coupling enzyme was observed in the absence of CoA. The assay contained (1 mL final volume) 60 μM crotonyl CoA, 50 μM Tris-Cl, pH 9, 50 μg bovine serum albumin per mL, 50 mM KCl, 1 mM NAD, 7 μg L-specific β-hydroxyacyl CoA dehydrogenase from porcine heart per mL, and 0.25 mM CoA. The assay was initiated with the addition of FaoAB to the assay mixture. A control assay was performed without substrate to determine the rate of consumption of NAD in the absence of the hydratase generated product, S-hydroxybutyryl CoA. One unit of activity is defined as the consumption of one μMol of NAD per min ($\varepsilon_{340} = 6220\ M^{-1}cm^{-1}$).

Hydroxyacyl CoA dehydrogenase was assayed in the reverse direction with acetoacetyl CoA as the substrate by monitoring the conversion of NADH to NAD at 340 nm (Binstock, et al., *Methods in Enzymology*, 71:403 (1981)). The assay contained (1 mL final volume) 0.1 M KH_2PO_4, pH 7, 0.2 mg bovine serum albumin per mL, 0.1 mM NADH, and 33 μM acetoacetyl CoA. The assay was initiated with the addition of FaoAB to the assay mixture. When necessary, enzyme samples were diluted in 0.1 M KH_2PO_4, pH 7, containing 1 mg bovine serum albumin per mL. A control assay was performed without substrate acetoacetyl CoA to detect the rate of consumption of NADH in the crude due to enzymes other than hydroxyacyl CoA dehydrogenase. One unit of activity is defined as the consumption of

one μMol of NADH per minute (ε_{340} = 6220 $M^{-1}cm^{-1}$).

HydroxyacylCoA dehydrogenase was assayed in the forward direction with crotonyl CoA as a substrate by monitoring the conversion of NAD to NADH at 340 nm (Binstock, et al., *Methods in Enzymology*, 71:403 (1981)). The assay mixture contained (1 mL final volume) 0.1 M KH_2PO_4, pH 8, 0.3 mg bovine serum albumin per mL, 2 mM β-mercaptoethanol, 0.25 mM CoA, 30 μM crotonyl CoA, and an aliquot of FaoAB. The reaction was preincubated for a couple of minutes to allow *in situ* formation of S-hydroxybutyryl CoA. The assay then was initiated by the addition of NAD (0.45 mM). A control assay was performed without substrate to detect the rate of consumption of NAD due to enzymes other than hydroxyacyl CoA dehydrogenase. One unit of activity is defined as the consumption of one μMol of NAD per minute (ε_{340} = 6220 $M^{-1}cm^{-1}$).

Thiolase activity was determined by monitoring the decrease in absorption at 304 nm due to consumption of substrate acetoacetyl CoA as previously described with some modifications (Palmer, et al., *J. Biol. Chem.* 266:1-7 (1991)). The assay contained (final volume 1 mL) 62.4 mM Tris-Cl, pH 8.1, 4.8 mM $MgCl_2$, 62.5 μM CoA, and 62.5 μM acetoacetyl CoA. The assay was initiated with the addition of FaoAB to the assay mixture. A control sample without enzyme was performed for each assay to detect the rate of substrate degradation of pH 8.1 in the absence of enzyme. One unit of activity is defined as the consumption of one μMol of substrate acetoacetyl CoA per minute (ε_{340} = 16900 $M^{-1}cm^{-1}$).

Epimerase activity was assayed as previously described (Binstock, et al., *Methods in Enzymology*, 71:403 (1981)) except that R-3-hydroxyacyl CoA thioesters were utilized instead of D,L-3-hydroxyacyl CoA mixtures. The assay contained (final volume 1 mL) 30 μM R-3-hydroxyacyl CoA, 150 mM KH_2PO_4 (pH 8), 0.3 mg/mL BSA, 0.5 mM NAD, 0.1 mM CoA, and 7 μg/mL L-specific β-hydroxyacyl CoA dehydrogenase from porcine heart. The assay was initiated with the addition of FaoAB.

For expression of FaoAB in DH5α/pMFX3, cultures were grown in 2xTY medium at 30 °C. 2xTY medium contains (per L) 16 g tryptone, 10 g

yeast, and 5 g NaCl. A starter culture was grown overnight and used to inoculate (1% inoculum) fresh medium (100 mL in a 250 mL Erlenmeyer flask for small scale growths; 1.5L in a 2.8L flask for large scale growths). Cells were induced with 0.4 mM IPTG when the absorbance at 600 nm was in the range of 0.4 to 0.6. Cells were cultured an additional 4 h prior to harvest. Cells were lysed by sonication, and the insoluble matter was removed from the soluble proteins by centrifugation. Acyl CoA dehydrogenase activity was monitored in the reverse direction to ensure activity of the FaoA subunit (SEQ ID NO:31) and thiolase activity was assayed to determine activity of the Fao subunit. FaoAB in DH5α/pMFX3 contained dehydrogenase and thiolase activity values of 4.3 and 0.99 U/mg, respectively, which is significantly more than the 0.0074 and 0.0033 U/mg observed for dehydrogenase and thiolase, respectively, in control strain DH5α/pTRCN.

FaoAB was purified from DH5α/pMFX3 using a modified procedure previously described for the purification of FaoAB from *Pseudomonas fragi* (Imamura, et al., *J. Biochem.* 107:184-89 (1990)). Thiolase activity (assayed in the forward direction) and dehydrogenase activities (assayed in the reverse direction) were monitored throughout the purification. Three liters of DH5α/pMFX3 cells (2 X 1.5 L aliquots in 2.8 L Erlenmeyer flasks) were grown in 2 x TY medium using the cell growth procedure previously described for preparing cells for enzyme activity analysis. Cells (15.8 g) were resuspended in 32 mL of 10 mM KH_2PO_4, pH 7, and lysed by sonication. Soluble proteins were removed from insoluble cells debris by centrifugation (18,000 RPM, 30 min., 4 °C). The soluble extract was made 50% in acetone and the precipitated protein was isolated by centrifugation and redissolved in 10 mM KH_2PO_4, pH 7. The sample was adjusted to 33% saturation with $(NH_4)_2SO_4$ and the soluble and insoluble proteins were separated by centrifugation. The resulting supernatant was adjusted to 56% saturation with $(NH_4)_2SO_4$ and the insoluble pellet was isolated by centrifugation and dissolved in 10 mM KH_2PO_4, pH 7. The sample was heated at 50°C for 30 min. and the soluble proteins were isolated by

centrifugation and dialyzed in a 6,000 to 8,000 molecular weight cut off membrane in 10 mM KH_2PO_4, pH 7 (2 X 3L; 20 h). The sample was loaded on a Toyo Jozo DEAE FPLC column (3 cm x 14 cm) that previously had been equilibrated in 10 mM KH_2PO_4, pH 7. The protein was eluted with a linear gradient (100 mL by 100 mL; 0 to 500 mM NaCl in 10 KH_2PO_4, pH 7) at a flow of 3 mL/min. FaoAB eluted between 300 and 325 mM NaCl. The sample was dialyzed in a 50,000 molecular weight cut off membrane in 10 mM KH_2PO_4, pH 7 (1 X 2L; 15h) prior to loading on a macro-prep hydroxylapatite 18/30 (Biorad) FPLC column (2 cm x 15 cm) that previously had been equilibrated in 10 mM KH_2PO_4, pH 7. The protein was eluted with a linear gradient (250 mL by 250 mL; 10 to 500 mM KH_2PO_4, pH 7) at a flow rate of 3 mL/min. FaoAB eluted between 70 and 130 mM KH_2PO_4. The fractions containing activity were concentrated to 9 mL using a MILLIPORE™ 100,000 molecular weight cutoff concentrator. The buffer was exchanged 3 times with 10 mM KH_2PO_4, pH 7 containing 20% sucrose and frozen at -70°C. Enzyme activities of the hydroxylapatite purified fraction were assayed with a range of substrates. The results are shown in Table 1 below.

Table 1: Enzyme Substrates and Activities

Enzyme	Substrate	Activity (U/mg)
hydratase	crotonyl CoA	8.8
dehydrogenase (forward)	crotonyl CoA	0.46
dehydrogenase (reverse)	acetoacetyl CoA	29
thiolase	acetoacetyl CoA	9.9
epimerase	R-3-hydroxyoctanyl CoA	0.022
epimerase	R-3- hydroxyhexanyl CoA	0.0029
epimerase	R-3- hydroxybutyryl CoA	0.000022

Example 2: Production of Antibodies to the FaoAB and FaoAB Polypeptides

Following purification of the FaoAB protein as described in Example 1, a sample was separated by SDS-PAGE. The protein band corresponding to the FaoA (SEQ ID NO:31) and FaoB (SEQ ID NO:26) was excised and used to immunize New Zealand white rabbits with complete Freunds adjuvant. Boosts were performed using incomplete Freunds at three week

intervals. Antibodies were recovered from serum by affinity chromatography on Protein A columns (Pharmacia) and tested against the antigen by Western blotting procedures. Control extracts of *Brassica* seeds were used to test for cross reactivity to plant proteins. No cross reactivity was detected.

Example 3: Construction of Plasmids for Expression of the *Pseudomonas putido fao* AB Genes in Transgenic Oilseeds

Construction of pSBS2024

Oligonucleotide primers GVR471

*5'-CGGTACC*CATTGTACTCCCAGTATCAT-3' (SEQ ID NO:8)

and GVR472

5'-*CATTTAAAT*AGTAGAGTATTGAATATG-3' (SEQ ID NO:9)

homologous to sequences flanking the 5' and 3' ends (underlined), respectively, of the bean phaseolin promoter (SEQ ID NO:10; Slightom *et al.*, 1983) were designed with the addition of *Kpn*I (in italics, nucleotides 1-7 in SEQ ID NO:8) and *Swa*I (in italics, nucleotides 1-9 in SEQ ID NO:9) at the 5' ends of GVR471 and GVR472, respectively. These restriction sites were incorporated to facilitate cloning. The primers were used to amplify a 1.4 kb phaseolin promoter, which was cloned at the *Sma*I site in pUC19 by blunt ended ligation. The designated plasmid, pCPP1 (see Figure 2) was cut with *Sal*I and *Swa*I and ligated to a *SalI/Swa*I phaseolin terminator (SEQ ID NO:27). The bean phaseolin terminator sequence encompassing the polyadenylation signals was amplified using the following PCR primers:

GVR396:

5'-GATTTAAATGCAAGCTTAAATAAGTATGAACTAAAATGC-3'

(SEQ ID NO:22)

and GVR397:

5'-CGGTACCTTAGTTGGTAGGGTGCTA-3' (SEQ ID NO.23)

and the 1.2Kb fragment (SEQ ID NO:27) cloned into Sal1-SaI site of pCCP1 to obtain pSBS2024 (Figure 2). The resulting plasmid which contains a unique *Hind*III site for cloning was called pSBS2024 (Figure 2).

Construction of pSBS2025

A soybean oleosin promoter fragment (SEQ ID NO:11; Rowley *et al.*, 1997) was simplified with primers that flank the DNA sequence.

Primer JA408

5' –*TCTAGA*TACATCCATTTCTTAATATAATCCTCTTATTC-3'

(SEQ ID NO:12)

contains sequences that are complementary to the 5' end (underlined).

Primer np1

5' –*CATTTAAAT*GGTTAAGGTGAAGGTAGGGCT-3'

(SEQ ID NO:13)

contains sequences homologous to the 3' end (underlined) of the promoter fragment. The restriction sites *Xbal* (in italics) and *Swa*I (in italics) were incorporated at the 5' end of JA408 and np1, respectively, to facilitate cloning. The primers were used to amplify a 975 bp promoter fragment, which then was cloned into *Sma*I site of pUC19 (see Figure 2). The resulting plasmid, pCSPI, was cut with *Sal*I and *Swa*I and ligated to the soybean terminator (SEQ ID NO:28). The soybean oleosin terminator was amplified by PCR using the following primers:

JA410:

5'-AAGCTTACGTGATGAGTATTAATGTGTTGTTATG-3'

(SEQ ID NO:29)

and

JA411:

5'-TCTAGACAATTCATCAAATACAAATCACATTGCC-3'

(SEQ ID NO:30)

and the 225 bp fragment cloned into the SalI-SwaI site of pCSP1 to obtain plasmid pSBS2025 (Figure 6). The designated plasmid, pSBS2025, carried a unique *Hind*III site for cloning (Figure 2).

Construction of Promoter-coding Sequence Fusions

Two oligonucleotide primers were synthesized:

np2

5'*AAGCTT*AAAATGATTTACGAAGGTAAAGCC-3'

(SEQ ID NO:14)

homologous to nucleotides 553 to 573 of the 5' flanking sequences, and

np3

5' *ATTGCTT*TCAGTTGAAGCGCTG-3'

(SEQ ID NO:15)

complementary to nucleotides 2700 to 2683 flanking the 3' end of mfl (faoA, SEQ ID NO:24) of plasmid pmfx3. A *Hind*III (in italics) site was introduced at the 5' end of primers np2 and np3 to facilitate cloning. In addition, a 3 bp AAA sequence (bold) was incorporated to obtain a more favorable sequence surrounding the plant translational initiation codon. Primers np2 and np3 were used to amplify the fragment and cloned into *Sma*I site of pUC19. The resulting plasmid was called pCmfl (Figures 3A and 3B). Plasmid pBmf2 was constructed in a similar process (Figures 5A and 5B). In order to generate a *Hind*III (in italics) at 5' and 3' ends of the mf2 (faoB) gene (SEQ ID NO:25) for cloning, a second set of synthetic primers were designed.

Primers np4

5' –*AAGCTTAAA*ATGAGCCTGAATCCAAGAGAC-3'

(SEQ ID NO:16)

complementary to 5' (nucleotides 2732-2752 bp) and np5

5'*AAGCTT*TCAGACGCGTTCGAAGACAGTG –3'

(SEQ ID NO:17)

homologous to 3' (nucleotides 3907-3886 bp) sequences of mf2 (faoB, SEQ ID NO:25) of plasmid pmfx3 were used in a PCR reaction to amplify the 1.17 kb DNA fragment. The resulting PCR product was cloned into the *EcoRV* site of pBluescript. The plasmid was referred to as pBmf2.

Both plasmids were individually cut with *Hind*III and their inserts cloned in plasmids pSBS2024 and pSBS2025, which had previously been linearized with the same restriction enzyme. As a result, the following

plasmids were generated: pmf124 and pmf125 (Figures 3A and 3B) and pmf224 and pmf225 (Figures 5A and 5B) containing the Fao genes (mf1 and mf2) fused to either the phaseolin or soybean promoters. DNA sequence analysis confirmed the correct promoter-coding sequence-termination sequence fusions for pmf124, pmf125, pmf224, and pmf225.

Example 4: Assembly of Promoter-coding Sequence Fusions into Plant Transformation Vectors

After obtaining plasmids pmf124, pmf125, pmf224, and pmf225, promoter-coding sequence fusions were independently cloned into the binary vectors, pCGN1559 (McBride and Summerfelt, 1990) containing the CaMV 35S promoter driving the expression of NPTII gene (conferring resistance to the antibiotic kanamycin) and pSBS2004 containing a parsley ubiquitin promoter driving the PPT gene, which confers resistance to the herbicide phosphinothricine. Binary vectors suitable for this purpose with a variety of selectable markers can be obtained from several sources.

The phaseolin-mf21 fusion cassette was released from the parent plasmid with *Xba*I and ligated with pCGN1559, which had been linearized with the same restriction enzyme. The resulting plasmid was designated pCGmf124 (Figures 3A and 3B). Plasmid pCGmf125 containing the soybean-mf1 fusion was constructed in a similar way (Figures 3A and 3B), except that both pmf125 and pCGN1559 were cut with *Bam*HI before ligation.

Construction of pmf1249 an pmf1254

The plasmid pSBS2004 was linearized with *Bam*HI fragment containing the soybean-mf1 fusion. This plasmid was designated pmf1254 (Figures 4A and 4B). Similarly, the XbaI phaseolin-mf1 fusion fragment was ligated to pSBS2004 which had been linearized with the same restriction enzyme. The resulting plasmid was designated pmf1249 (Figures 4A and 4B).

Construction of pCGmf224 and pCGmf225

The phaseolin-mf2 and soybean-mf2 fusions were constructed by

excising the fusions from the vector by cutting with either *Bam*HI or *Xba*I, and cloned into pCGN1559 which had been linearized with either restriction enzyme (Figures 5A and 5B).

Construction of pCGmf1P2S and pCGmf2P1S

The two expression cassettes containing the promoter-coding sequence fusions were assembled on the same binary vector as follows: Plasmid pmf124 containing the phaseolin-mf1 fusion was cut with *Bam*HI and cloned into the *Bam*HI site of pCGN1559 to create pCGmfB124. This plasmid then was linearized with *Xba*I and ligated to the *Xba*I fragment of pmf225 containing the soybean-mf2 fusion. The final plasmid was designated pCGmf1P2S (Figures 6A and 6B). Plasmid pCGmf2P1S was assembled in similar manner. The phaseolin-mf2 fusion was released from pmf224 by cutting with *Bam*HI and cloned at the *Bam*HI site of pCGN1559. The resulting plasmid, pCGmfB224, was linearized with *Xba*I and ligated to the *Xba*I fragment of pmf125 containing the soybean-mf1 fusion (Figures 6A and 6B).

Example 5: Transformation of *Brassica*

Brassica seeds were surface sterilized in 10% commercial bleach (Javex, Colgate-Palmolive) for 30 min. with gentle shaking. The seeds were washed three times in sterile distilled water. Seeds were placed in germination medium comprising Murashige-Skoog (MS) salts and vitamins, 3% (w/v) sucrose and 0.7% (w/v) phytagar, pH 5.8 at a density of 20 per plate and maintained at 24 °C and a 16 h light / 8 h dark photoperiod at a light intensity of 60-80 $\mu Em^{-2}s^{-1}$ for four to five days.

Each of the constructs, pCGmf124, pCGmf125, pCGmf224, pCGmf1P2S, and pCGmf2P1S were introduced into *Agrobacterium tumefacians* strain EHA101 (Hood et al., *J. Bacteriol.* 168:1291-1301 (1986)) by electroporation. Prior to transformation of cotyledonary petioles, single colonies of strain EHA101 harboring each construct were grown in 5 ml of minimal medium supplemented with 100 mg kanamycin per liter and 100 mg gentamycin per liter for 48 hr at 28 °C. One milliliter of bacterial

suspension was pelletized by centrifugation for 1 min in a microfuge. The pellet was resuspended in 1 ml minimal medium.

For transformation, cotyledons were excised from 4 day old, or in some cases 5 day old, seedlings, so that they included approximately 2 mm of petiole at the base. Individual cotyledons with the cut surface of their petioles were immersed in diluted bacterial suspension for 1 s and immediately embedded to a depth of approximately 2 mm in co-cultivation medium, MS medium with 3% (w/v) sucrose and 0.7% phytagar and enriched with 20 μM benzyladenine. The inoculated cotyledons were plated at a density of 10 per plate and incubated under the same growth conditions for 48 h. After co-cultivation, the cotyledons then were transferred to regeneration medium comprising MS medium supplemented with 3% sucrose, 20 μM benzyladenine, 0.7% (w/v) phytagar, pH 5.8, 300 mg timentinin per liter, and 20 mg kanamycin sulfate per liter.

After two to three weeks, regenerant shoots obtained were cut and maintained on "shoot elongation" medium (MS medium containing, 3% sucrose, 300 mg timentin per liter, 0.7% (w/v) phytagar, 300 mg timentinin per liter, and 20 mg kanamycin sulfate per liter, pH 5.8) in Magenta jars. The elongated shoots were transferred to "rooting" medium comprising MS medium, 3% sucrose, 2 mg indole butyric acid per liter, 0.7% phytagar, and 500 mg carbenicillin per liter. After roots emerged, plantlets were transferred to potting mix (Redi Earth, W.R. Grace and Co.). The plants were maintained in a misting chamber (75% relative humidity) under the same growth conditions. Two to three weeks after growth, leaf samples were taken for neomycin phosphotransferase (NPTII) assays (Moloney et al., *Plant Cell Reports* 8:238-42 (1989)).

Seeds from the FaoA and FaoB transgenic lines can be analyzed for expression of the fatty acid oxidation polypeptides by western blotting using the anti-FaoA and anti-FaoB antibodies. The FaoB polypeptide (SEQ ID NO:26) is not functional in the absence of the FaoA gene product; however, the FaoAB gene product has enzyme activity.

Transgenic lines expressing the FaoA and FaoB complex are obtained

by crossing the FaoA and FaoB transgenic lines expressing the individual polypeptides and seeds analyzed by western blotting and enzymes assays as described.

Example 6: Transformation of *B. napus* cv. Westar and Analysis of Transgenic Lines

Transformation

The protocol used was adopted from a procedure described by Moloney *et al.* (1989). Seeds of *Brassica napus* cv. Westar were surface sterilized in 10% commercial bleach (Javex, Colgate-Palmolive Canada Inc.) for 30 min with gentle shaking. The seeds were washed three times in sterile distilled water. Seeds were placed on germination medium comprising Murashige-Skoog (MS) salts and vitamins, 3% sucrose and 0.7% phytagar, pH 5.8 at a density of 20 per plate and maintained at 24 °C in a 16 h light/8 h dark photoperiod at a light intensity of 60-80 $\mu Em^{-2}s^{-1}$ for four to five days.

Each of the constructs, pCGmf124, pCGmf125, pCGmf224, pCGmf225, pCGmf1P2S, and pCGmf2P1S were introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.* 1986) by electroporation. Prior to transformation of cotyledonary petioles, single colonies of strain EHA101 harboring each construct were grown in 5 mL of minimal medium supplemented with 100 mg kanamycin per liter, and 100 mg gentamycin per liter for 48 h at 28 °C. One milliliter of bacterial suspension was pelletized by centrifugation for 1 min in a microfuge. The pellet was resuspended in 1 mL minimal medium.

For transformation, cotyledons were excised from four-day-old, or in some cases five-day-old, seedlings so that they included approximately 2 mm of petiole at the base. Individual cotyledons with the cut surface of their petioles were immersed in diluted bacterial suspension for 1 s and immediately embedded to a depth of approximately 2 mm in co-cultivation medium, MS medium with 3% sucrose and 0.7% phytagar, enriched with 20 μM benzyladenine. The inoculated cotyledons were plated at a density of 10 per plate and incubated under the same growth conditions for 48 h. After

co-cultivation, the cotyledons then were transferred to regeneration medium, which comprised MS medium supplemented with 3% sucrose, 20 μM benzyladenine, 0.7% phytagar, pH 5.8, 300 mg timentinin per liter, and 20 mg kanamycin sulfate per liter.

After two to three weeks, regenerant shoots were obtained, cut, and maintained on "shoot elongation" medium (MS medium containing 3% sucrose, 300mg timentin per liter, 0.7% phytagar, and 20 mg kanamycin per liter, pH 5.8) in Magenta jars. The elongated shoots then were transferred to "rooting" medium, which comprised MS medium, 3% sucrose, 2 mg indole butyric acid per liter, 0.7% phytagar and 500 mg carbenicillin per liter. After roots emerged, the plantlets were transferred to potting mix (Redi Earth, W.R. Grace and Co. Canada Ltd.). The plants were maintained in a misting chamber (75% RH) under the same growth conditions. Two to three weeks after growth, leaf samples were taken for neomycin phosphotransferase (NPT II) assays (Moloney *et al.* 1989). The results are shown in Table 2 below. The data show the number of plants that were confirmed to be transformed.

Table 2: NPT II Activity in Transformed Plants

Constructs	No. of plants	NPTII assayed	NPTII confirmed	No. of plants confirmed transformed
[1]pCGmf124	47	27	23	33
[2]pCGmf125	37	28	18	18
[3]pCGmf224	49	40	30	39
[4]pCGmf225	52	37	28	34
[5]pCGmf1P2S [6]pCGmf2P1S	27	27	21	21

[1]pCGmf124 – bean phaseolin regulatory sequences driving FaoA gene
[2]pCGmf125 – soybean oleosin regulatory sequences driving FaoA gene
[3]pCGmf224 – bean phaseolin regulatory sequences driving FaoB gene
[4]pCGmf225 – soybean oleosin regulatory sequences driving FaoB gene
[5]pCGmf192S – bean phaseolin and soybean oleosin regulatory sequences driving FaoA & FaoB genes, respectively
[6]pCGmf2P1S – bean phaseolin and soybean oleosin regulatory sequences driving FaoB & FaoA genes, respectively

The fate of the transforming DNA was investigated for sixteen randomly selected transgenic lines. Southern DNA hybridization analysis showed that the FaoA and/or FaoB were integrated into the genomes of the

transgenic lines tested.

Approximately 80% of the pmf124 transgenic plants in which the FaoA gene is expressed from the strong bean phaseolin promoter were observed to be male sterile. Clearly high level expression of the FaoA gene from this promoter results in functional expression of the FaoA gene product which impairs seed and/or pollen development. This result was very unexpected, since it was not anticipated that the plant cells would be capable of carrying out the first step in the β-oxidation pathway in the cytosol. This result, however, provides additional applications for expressing β-oxidation genes in plants for male sterility for hybrid production or to prevent the production of seed. It was also note that in a side-by-side comparison with normal transgenic lines, the pmf124 lines produced much higher levels of biomass, presumably due to the elimination of seed development. This phenotype therefore may be useful as a means to increase the amount of green biomass produced per acre for silage, forage, or other biomass crops. Here, the use of an inducible promoter system or recombinase technology could be used to produce seed for planting. Seven of the sterile plants were successfully cross-pollinated with pollen from pmf225 transgenic lines and set seeds.

Northern analysis on RNA from seeds from pmf224 lines containing the phaseolin promoter-FaoB constructs showed a signal indicative of the expected 1.2 kb transcript in all the samples tested except the control. Northern analysis on RNA from seeds from pmf125 lines containing the weak soybean oleolsin promoter-FaoA constructs revealed a transcript of the expected size of 2.1 kb. Western blotting on 300-500 μg of protein from approximately 80% of seeds of pmf125 plants where the FaoA gene is expressed from the relatively weak soybean oleosin promoter were inconclusive, although a weak signal was detected in one transgenic line.

Fatty Acid Analysis

Given the unexpected results indicating a strong metabolic effect of expressing the FaoA gene from the strong bean phaseolin promoter in seeds, the fatty acid profile of the seeds from transgenic lines expressing the FaoA

gene from the weak soybean oleosin promoter was analyzed. Seeds expressing only the FaoA gene or also expressing the FaoB gene from the bean phaseolin promoter were examined. The analysis was carried out as described in Millar et al., *The Plant Cell* 11:1889-902 (1998). Seed fatty acid methyl esters (FAMES) were prepared by placing ten seeds of *B. napus* in 15 x 45-mm screw capped glass tubes and heating at 80 °C in 0.75 mL of 1N methanolic HCl reagent (Supelco, PA) and 10 µL of 1 mg 17:0 methyl ester (internal standard) per mL overnight. After cooling the samples, the FAMES were extracted with 0.3 mL hexane and 0.5 mL 0.9% NaCl by vortexing vigorously. The samples were allowed to stand to separate the phases, and 300 µL of the organic phase was drawn and analyzed on a Hewlett-Packard gas chromatograph.

Fatty acid profile analysis indicated the presence of an additional component or enhanced component in the lipid profile in all of the transgenic plants expressing the FaoA gene SEQ ID NO:24 which was absent from the control plants. This result again proves conclusively that the FaoA gene is being transcribed and translated and that the FaoA polypeptide SEQ ID NO: 27 is catalytically active. This peak also was observed in eleven additional transgenic plants harboring SoyP-FaoA, PhaP-FaoA-SoyP-FaoB, SoyP-FaoA-PhaP-FaoB genes and a sterile (PhaP-FaoA) plant cross-pollinated with SoyP-FaoB. These data clearly demonstrate functional expression of the FaoA gene and that even the very low levels of expression are sufficient to change the lipid profile of the seed. Adapting the methods described herein, one of skill in the art can express these genes at levels intermediate between that obtained with the phaseolin promoter and the soybean oleosin promoter using other promoters such as the *Arabidopsis oleosin* promoter, napin promoter, or cruciferin promoter, and can use inducible promoter systems or recombinase technologies to control when fatty acid oxidation transgenes are expressed.

Example 7: Yeast β-oxidation Multi-functional Enzyme Complex

S. cerevisiae contains a β-oxidation pathway that proceeds via R-

hydroxyacyl CoA rather than the S-3-hydroxyacyl CoA observed in bacteria and higher eukaryotes. The *fox2* gene from yeast encodes a hydratase that produces R-3-hydroxyacyl CoA from *trans*-2-enoyl-CoA and a dehydrogenase that utilizes R-3-hydroxyacyl-CoA to produce β-keto acyl CoAs.

The *fox2* gene (sequence shown in SEQ ID NO:1) was isolated from *S. cerevisiae* genomic DNA by PCR in two pieces. Primers N-fox2b and N-bamfox2b were utilized to PCR a 1.1 kb SmaI/BamHI fragment encoding the N-terminal region of Fox2, and primers C-fox2 and C-bamfox2 were utilized to PCR a 1.6 kb BamHI/XbaI fragment encoding the C-terminal Fox2 region. The full *fox2* gene was reconstructed via subcloning in vector pTRCN.

N-fox2b fox2

tcc ccc ggg agg agg ttt tta tta tgc ctg gaa att tat cct tca aag ata gag tt (SEQ ID NO:18)

N-bamfox2b fox2

aaggatccttgatgtcatttacaactacc (SEQ ID NO:19)

C-fox2 fox2

gct cta gat agg gaa aga tgt atg taa g (SEQ ID NO:20)

C-bamfox2 fox2

tgacatcaaggatcctttt (SEQ ID NO:21)

The *fox1* gene, however, does not possess a β-ketothiolase activity and this activity must be supplied by a second transgene. Representative sources of such a gene include algae, bacteria, yeast, plants, and mammals. The bacterium *Alcaligenes eutrophus* possesses a broad specificity β-ketothiolase gene suitable for use in the methods described herein. It can be readily isolated using the acetoacetyl-CoA thiolase gene as a hybridization probe, as described in U.S. Patent 5,661,026 to Peoples et al. This enzyme also has been purified (Haywood et al., *FEMS Micro. Lett.* 52:91 (1988)), and the purified enzyme is useful for preparing antibodies or determining protein sequence information as a basis for the isolation of the gene.

Example 8: Plant β-Oxidation Gene

The DNA sequence of the cDNA encoding β-oxidation tetrafunctional protein, shown in SEQ ID NO:4, can be isolated as described in Preisig-Muller et al., *J. Biol. Chem.* 269:20475-81 (1994). The equivalent gene can be isolated from other plant species including *Arabidopsis, Brassica,* soybean, sunflower, and corn using similar procedures or by screening genomic libraries, many of which are commercially available, for example from Clontech Laboratories Inc., Palo Alto, California, USA. A peroxisomal targeting sequence P-R-M was identified at the carboxy terminus of the protein. Constructs suitable for expressing in the plant cytosol can be prepared by PCR amplification of this gene using primers designed to delete this sequence.

We claim:

1. A method for manipulating the metabolism of a plant, comprising

expressing heterologous genes encoding fatty acid oxidation enzymes in cytosol or plastids other than the peroxisomes, glyoxisomes or mitochondria of the plant.

2. The method of claim 1 wherein the fatty acid β-oxidation enzymes are expressed from genes selected from the group consisting of bacterial, yeast, fungal, plant, and mammalian.

3. The method of claim 2 wherein the fatty acid oxidation enzymes are expressed from genes from bacteria selected from the group consisting of *Escherichia, Pseudomonas, Alcaligenes,* and *Coryneform.*

4. The method claim 3 wherein the genes are *Pseudomonas putida fao*AB.

5. The method of claim 1 further comprising

expressing genes encoding enzymes selected from the group consisting of polyhydroxyalkanoate synthases, acetoacetyl-CoA reductases, β-ketoacyl-CoA thiolases, and enoyl-CoA hydratases.

6. A DNA construct for use in a method of manipulating the metabolism of a plant cell comprising, in phase,

(a) a promoter region functional in a plant;

(b) a structural DNA sequence encoding at least one fatty acid oxidation enzyme activity; and

(c) a 3' nontranslated region of a gene naturally expressed in a plant, wherein the nontranslated region encodes a signal sequence for polyadenylation of mRNA.

7. The DNA construct of claim 6 wherein the promoter is a seed specific promoter.

8. The DNA construct of claim 7 wherein the seed specific promoter is selected from the group consisting of napin promoter, phaseolin promoter, oleosin promoter, 2S albumin promoter, zein promoter, β-conglycinin promoter, acyl-carrier protein promoter, and fatty acid desaturase promoter.

9. The DNA construct of claim 6 wherein the promoter is a constitutive promoter.

10. The DNA construct of claim 6 wherein the promoter is selected from the group consisting of CaMV 35S promoter, enhanced CaMV 35S promoter, and ubiquitin promoter.

11. A method for enhancing the biological production of polyhydroxyalkanoates in a transgenic plant, comprising

expressing genes encoding heterologous fatty acid oxidation enzymes in cytosol or plastids other than the peroxisomes, glyoxisomes or mitochondria of the plant.

12. The method of claim 11 wherein the transgenic plant is selected from the group consisting of *Brassica*, maize, soybean, cottonseed, sunflower, palm, coconut, safflower, peanut, mustards, flax, tobacco, and alfalfa.

13. A transgenic plant or part thereof comprising

heterologous genes encoding fatty acid oxidation enzymes in cytosol or plastids other than the peroxisomes, glyoxisomes or mitochondria of the plant.

14. The plant or part thereof of claim 13 wherein the fatty acid β-oxidation enzymes are expressed from genes selected from the group consisting of bacterial, yeast, fungal, plant, and mammalian.

15. The plant or part thereof of claim 14 wherein the fatty acid oxidation enzymes are expressed from genes from bacteria selected from the group consisting of *Escherichia, Pseudomonas, Alcaligenes,* and *Coryneform*.

16. The plant or part thereof of claim 15 wherein the genes are *Pseudomonas putida fao*AB.

17. The plant or part thereof of claim 13 further comprising

genes encoding enzymes selected from the group consisting of polyhydroxyalkanoate synthases, acetoacetyl-CoA reductases, β-ketoacyl-CoA thiolases, and enoyl-CoA hydratases.

18. The plant or part thereof of claim 13 wherein the plant is selected from the group consisting of *Brassica*, maize, soybean, cottonseed, sunflower, palm, coconut, safflower, peanut, mustards, flax, tobacco, and alfalfa.

19. The plant or part thereof comprising a DNA construct comprising, in phase,

(a) a promoter region functional in a plant;

(b) a structural DNA sequence encoding at least one fatty acid oxidation enzyme activity; and

(c) a 3' nontranslated region of a gene naturally expressed in a plant, wherein the nontranslated region encodes a signal sequence for polyadenylation of mRNA.

20. The plant or part thereof of claim 19 wherein the promoter is a seed specific promoter.

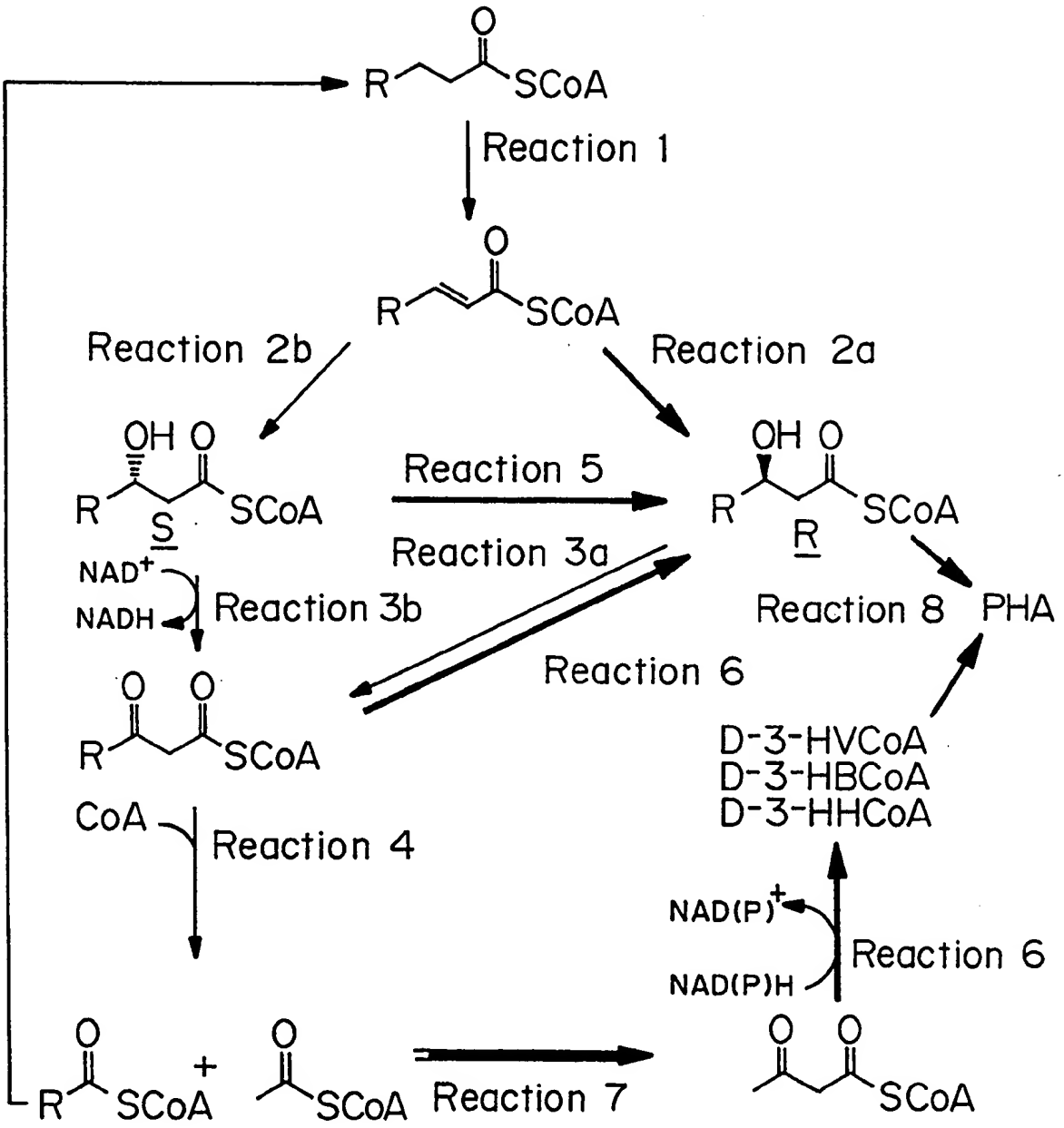
21. The plant or part thereof of claim 20 wherein the seed specific promoter is selected from the group consisting of napin promoter, phaseolin promoter, oleosin promoter, 2S albumin promoter, zein promoter, β-conglycinin promoter, acyl-carrier protein promoter, and fatty acid desaturase promoter.

22. The plant or part thereof of claim 19 wherein the promoter is a constitutive promoter.

23. The plant or part thereof of claim 20 wherein the promoter is selected from the group consisting of CaMV 35S promoter, enhanced CaMV 35S promoter, and ubiquitin promoter.

24. A method of preventing or suppressing seed production in a plant, comprising

expressing heterologous genes encoding fatty acid oxidation enzymes in cytosol or plastids other than the peroxisomes, glyoxisomes or mitochondria of the plant.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R
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Reaction 8
PHA
Reaction 6
CoA
Reaction 4
D-3-HVCoA
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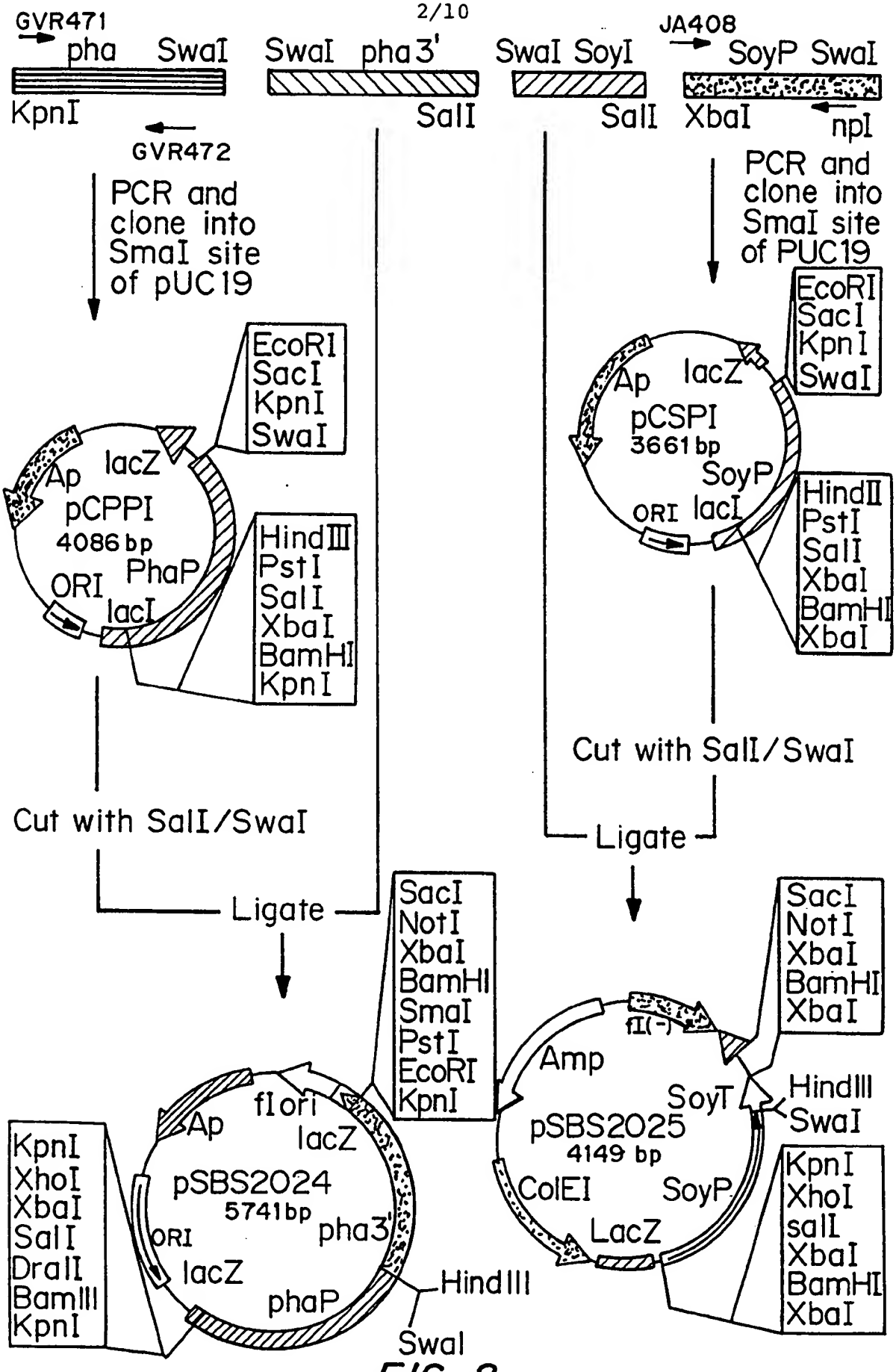
NAD(P)H
Reaction 6
Reaction 7

FIG. 1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PCR and clone into SmaI site of pUC19
PCR and clone into SmaI site of PUC19
EcoRI SacI KpnI SwaI
Ap lacZ pCPPI 4086 bp PhaP ORI lacI
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HindII PstI SalI XbaI BamHI XbaI
Cut with SalI/SwaI
Cut with SalI/SwaI
Ligate
Ligate
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f1ori
Ap lacZ
pSBS2024 5741bp
ORI
lacZ
pha3'
phaP
HindIII
SwaI
KpnI XhoI XbaI SalI DraII BamIII KpnI
f1(-)
Amp
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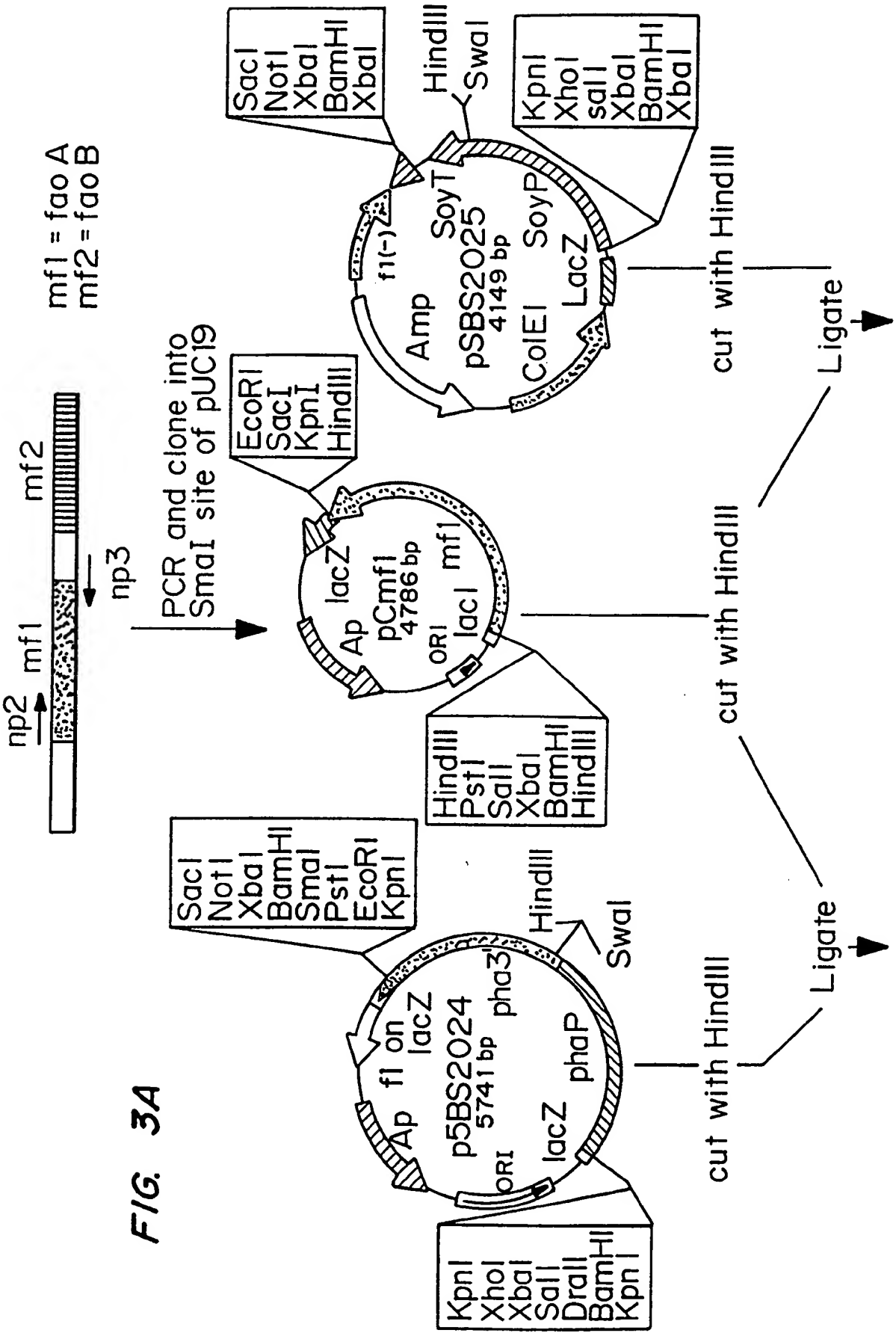
FIG. 2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mf2 = fao B
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mf1
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KpnI
HindIII
pCmf1
4786 bp
Ap
lacZ
ori
lacI
mf1
HindIII
PstI
SalI
XbaI
BamHI
HindIII
SacI
NotI
XbaI
BamHI
SmaI
PstI
EcoRI
KpnI
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SwaI
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lacZ
ori
lacZ
phaP
pha3
KpnI
XhoI
XbaI
SalI
DraII
BamHI
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SoyP
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Ligate
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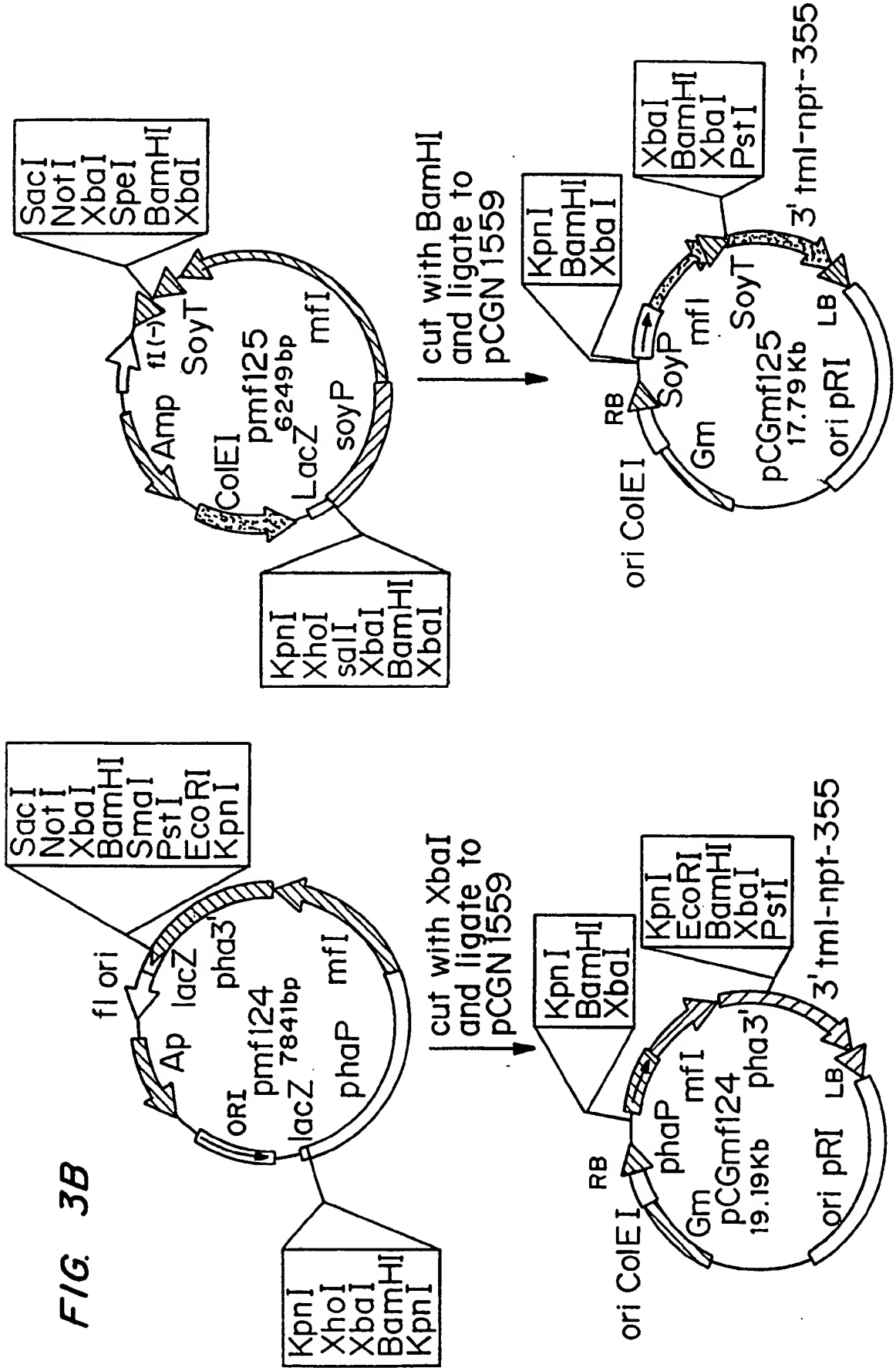
Ligate
cut with HindIII

FIG. 3A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and ligate to
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KpnI
BamHI
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3' tml-npt-355
RB
SoyP
mfI
SoyT
Gm
pCGmf125
17.79Kb
LB
ori ColE1
ori pRI
SacI
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XbaI
BamHI
SmaI
PstI
EcoRI
KpnI
f1 ori
Ap
lacZ
pha3'
ORI
pmf124
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BamHI
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and ligate to
pCGN1559
KpnI
BamHI
XbaI
KpnI
EcoRI
BamHI
XbaI
PstI
3' tml-npt-355
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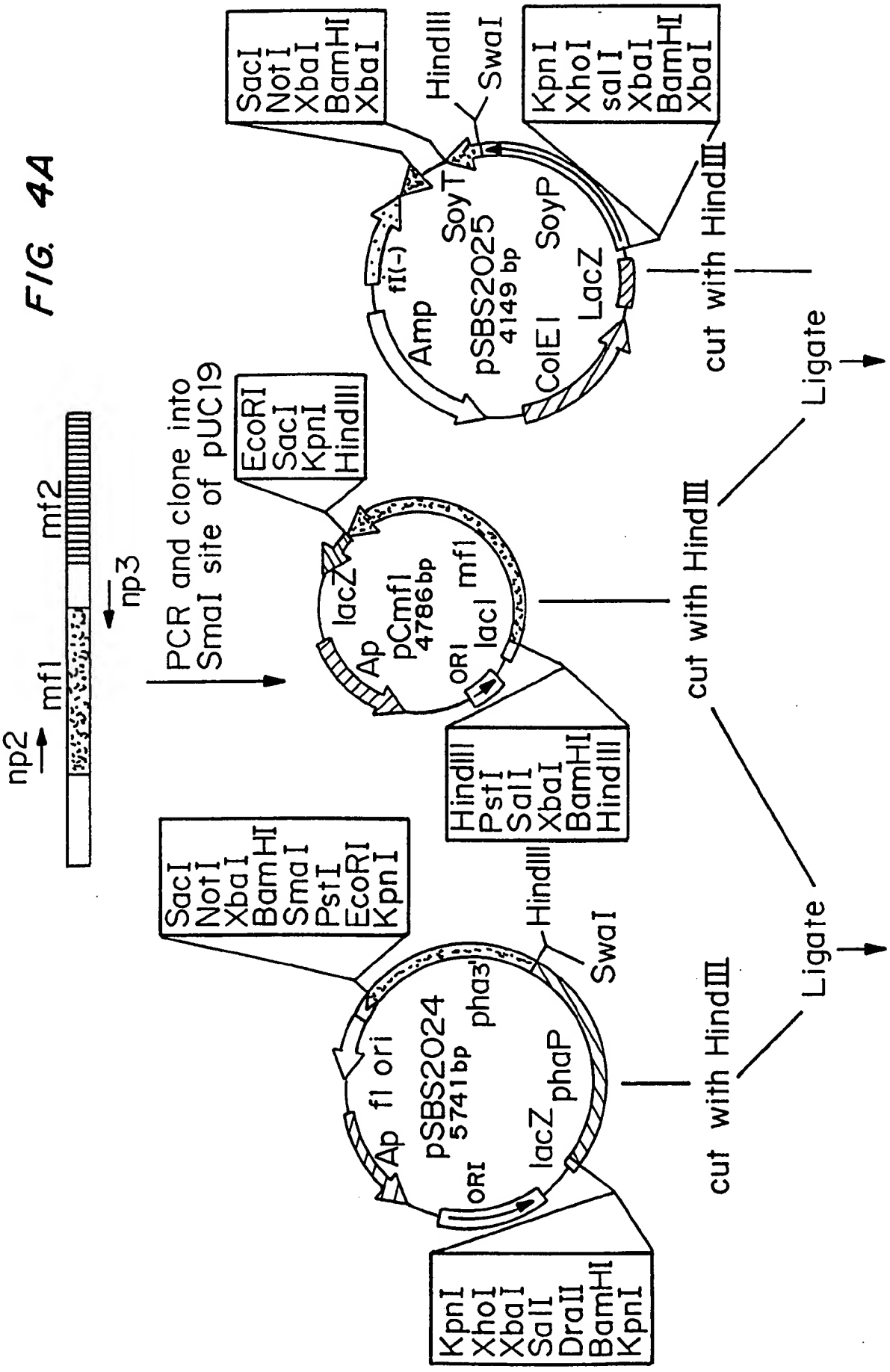
phaP
mfI
Gm
pCGmf124
19.19Kb
pha3'
LB
ori ColE1
ori pRI

FIG. 3B

FIG. 4A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pCmf1 4786bp
ORI
lacI
mf1
HindIII PstI SalI XbaI BamHI HindIII
SacI NotI XbaI BamHI SmaI PstI EcoRI KpnI
Ap f1 ori
pSBS2024 5741bp
ORI
pha3'
lacZ
phaP
HindIII
SwaI
KpnI XhoI XbaI SalI DraII BamHI KpnI
cut with HindIII
Ligate
cut with HindIII
Ligate
SacI NotI XbaI BamHI XbaI
HindIII
SwaI
KpnI XhoI salI XbaI BamHI XbaI
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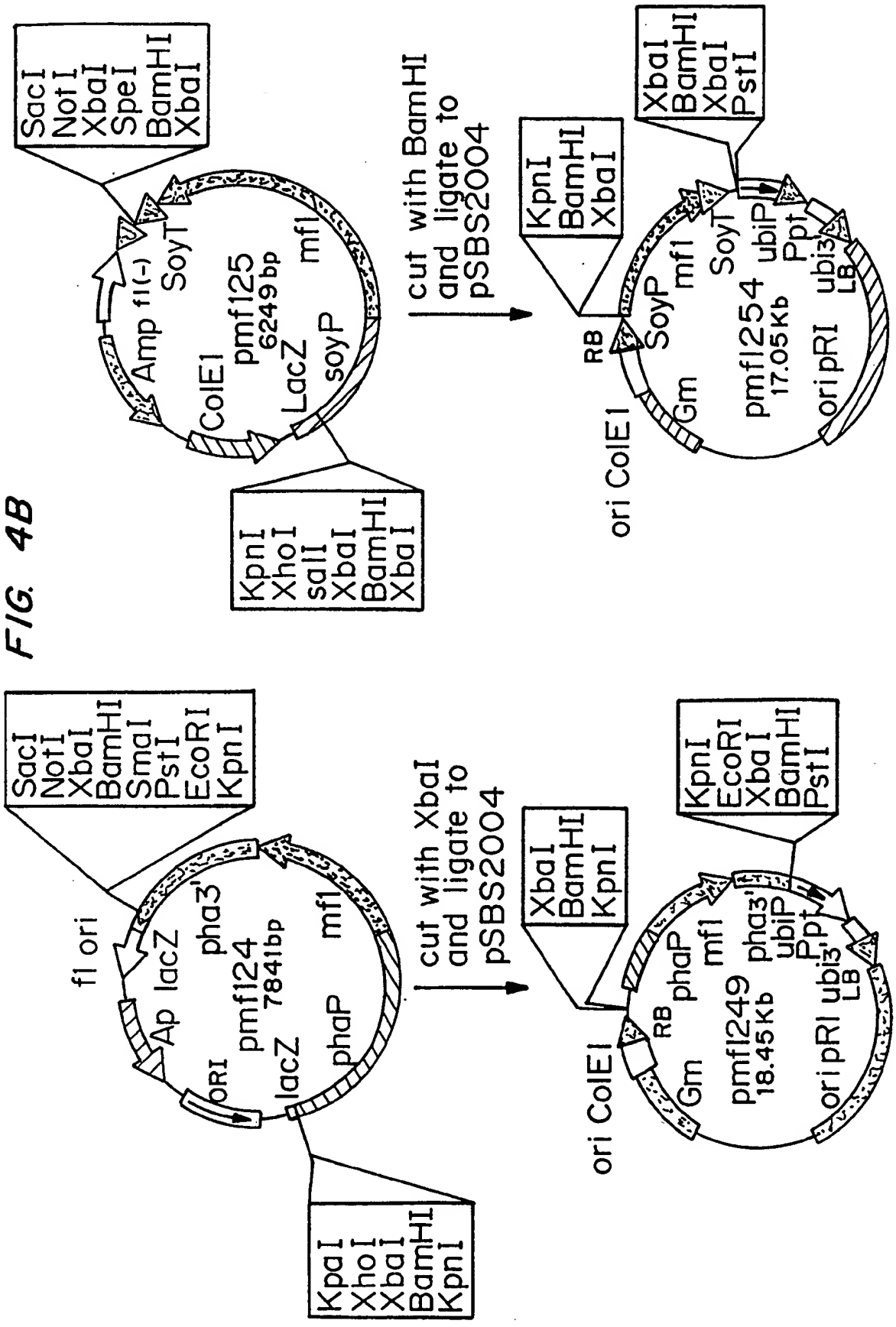
cut with HindIII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SoyT
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pmf125
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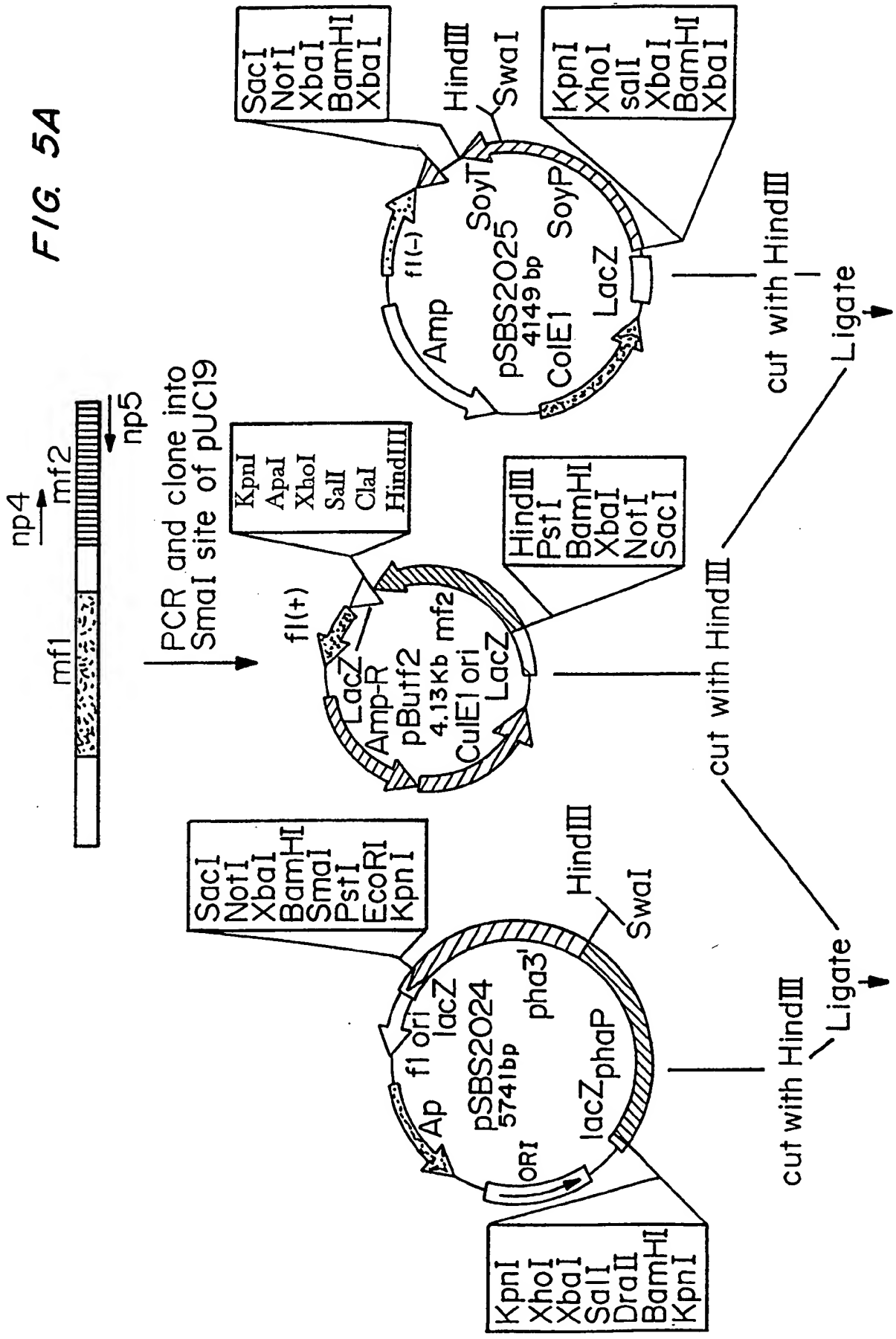
ColE1
LacZ
soyP
KpnI
XhoI
salI
XbaI
BamHI
XbaI
cut with BamHI
and ligate to
pSBS2004
KpnI
BamHI
XbaI
XbaI
BamHI
XbaI
PstI
RB
SoyP
mf1
SoyT
ubiP
Ppt
Gm
pmf1254
17.05Kb
oriPRI
ubi3'
LB
ori ColE1
SacI
NotI
XbaI
BamHI
SmaI
PstI
EcoRI
KpnI
f1 ori
Ap
lacZ
pha3'
pmf124
7841bp
mf1
ORI
lacZ
phaP
KpaI
XhoI
XbaI
BamHI
KpnI
cut with XbaI
and ligate to
pSBS2004
XbaI
BamHI
KpnI
KpnI
EcoRI
XbaI
BamHI
PstI
RB
phaP
mf1
pha3'
ubiP
Ppt
Gm
pmf1249
18.45Kb
oriPRI
ubi3'
LB
ori ColE1

FIG. 4B

FIG. 5A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mf2
np5
mf1
PCR and clone into
SmaI site of pUC19
KpnI
ApaI
XboI
SalI
ClaI
HindIII
f1(+)
LacZ
Amp-R
pButf2
4.13Kb
mf2
CulE1 ori
LacZ
HindIII
PstI
BamHI
XbaI
NotI
SacI
cut with HindIII
Ligate
SacI
NotI
XbaI
BamHI
SmaI
PstI
EcoRI
KpnI
HindIII
SwaI
f1 ori
lacZ
Ap
pSBS2024
5741bp
ORI
pha3'
lacZphaP
KpnI
XhoI
XbaI
SalI
DraII
BamHI
KpnI
cut with HindIII
Ligate
SacI
NotI
XbaI
BamHI
XbaI
HindIII
SwaI
f1(-)
SoyT
SoyP
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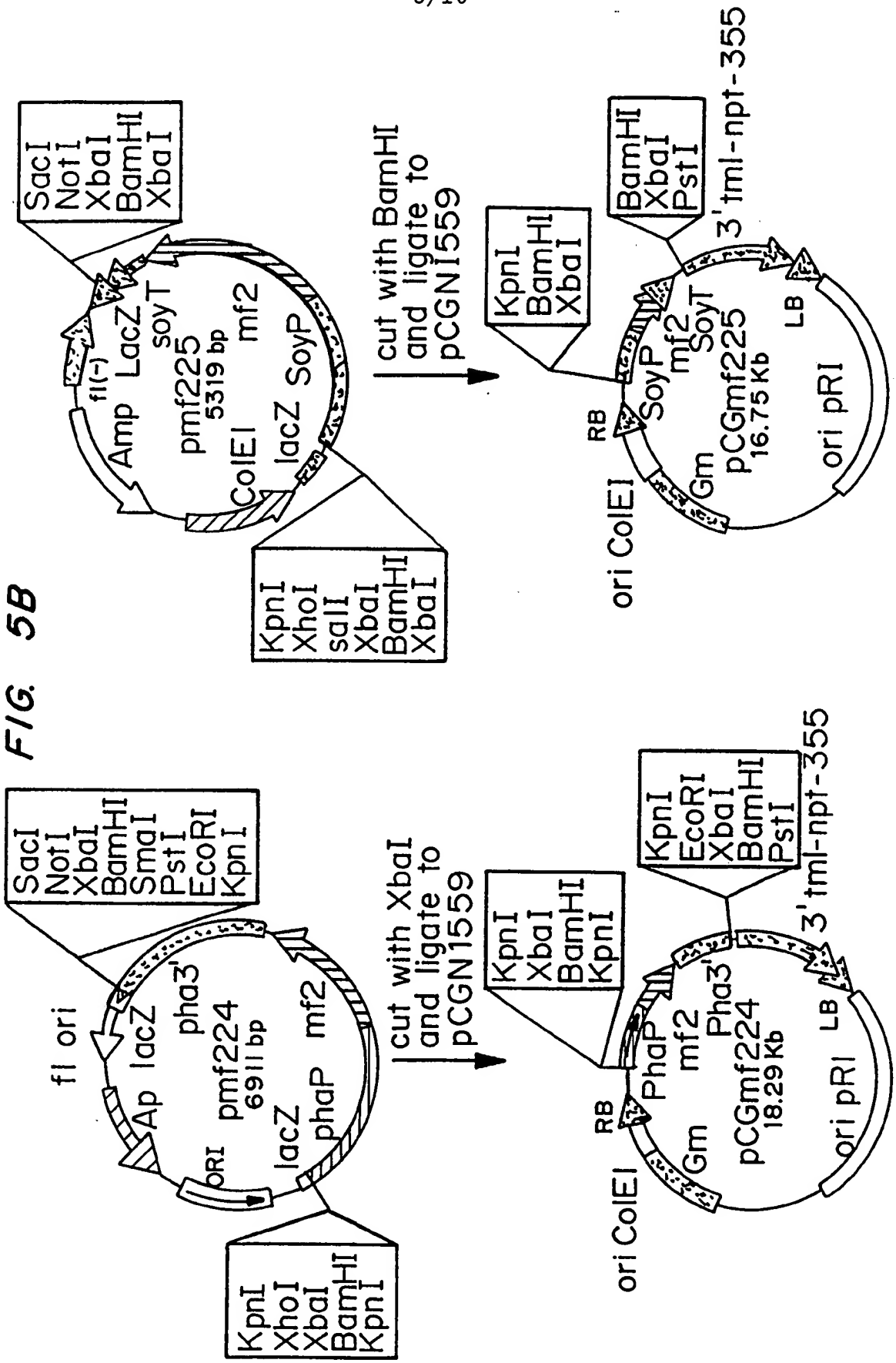
XhoI
salI
XbaI
BamHI
XbaI

FIG. 5B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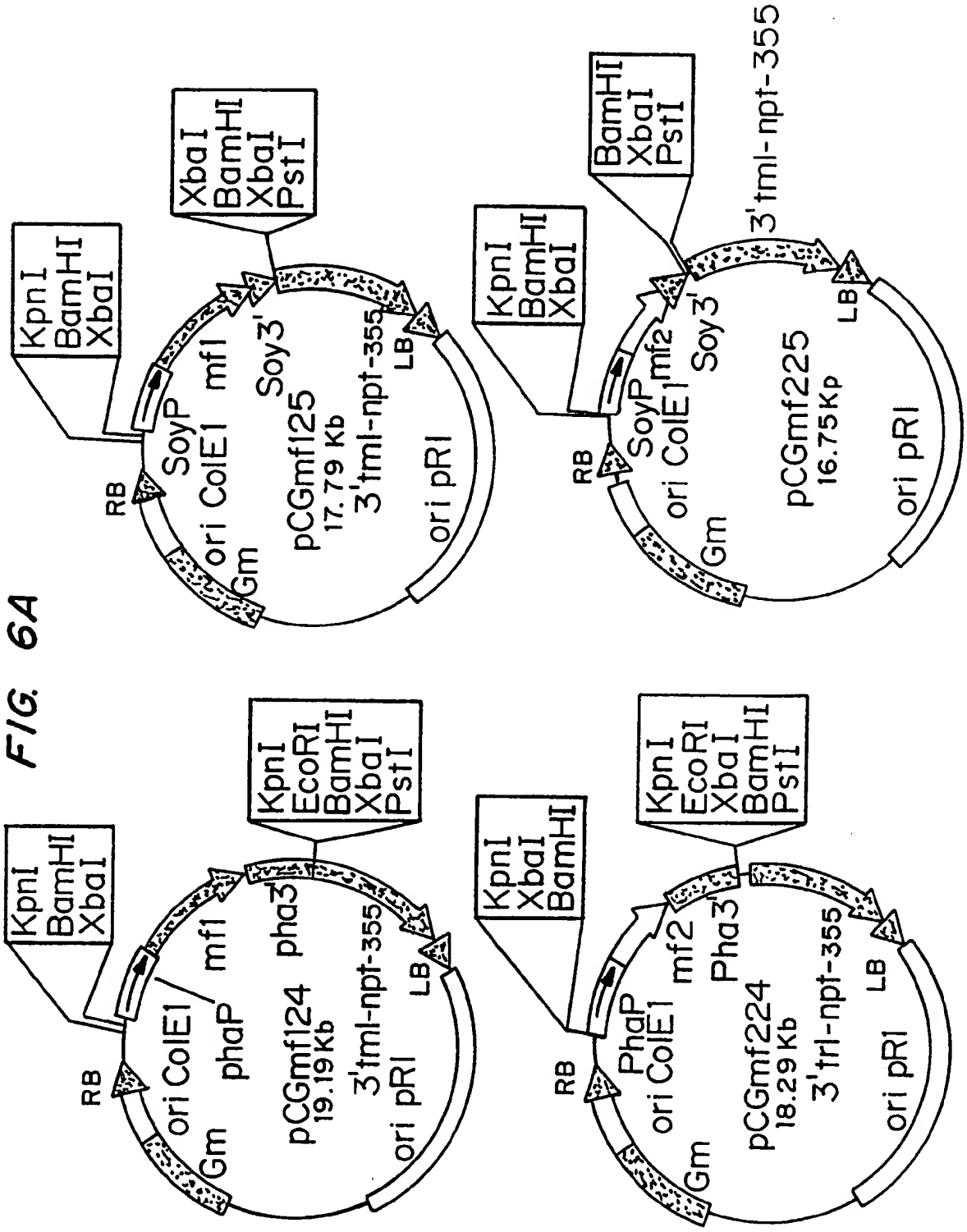
NotI
XbaI
BamHI
XbaI
pmf225
5319 bp
Amp
LacZ
soyT
mf2
ColE1
lacZ
SoyP
f1(-)
KpnI
XhoI
sall
XbaI
BamHI
XbaI
cut with BamHI
and ligate to
pCGN1559
KpnI
BamHI
XbaI
BamHI
XbaI
PstI
3' tml-npt-355
SoyP
mf2
SoyT
pCGmf225
16.75 Kb
RB
LB
Gm
ori ColE1
ori pRI
SacI
NotI
XbaI
BamHI
SmaI
PstI
EcoRI
KpnI
f1 ori
Ap
lacZ
pha3'
pmf224
6911 bp
mf2
phaP
oRI
lacZ
KpnI
XhoI
XbaI
BamHI
KpnI
cut with XbaI
and ligate to
pCGN1559
KpnI
XbaI
BamHI
KpnI
KpnI
EcoRI
XbaI
BamHI
PstI
3' tml-npt-355
PhaP
mf2
Pha3'
pCGmf224
18.29 Kb
RB
LB
Gm
ori ColE1
ori pRI

FIG. 6A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XbaI
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PstI
RB
SoyP
ori ColE1
mf1
Soy3'
pCGmf125
17.79 Kb
3'tml-npt-355
LB
Gm
ori pRI
KpnI
BamHI
XbaI
BamHI
XbaI
PstI
RB
SoyP
ori ColE1
mf2
Soy3'
pCGmf225
16.75 Kp
3'tml-npt-355
LB
Gm
ori pRI
KpnI
BamHI
XbaI
KpnI
EcoRI
BamHI
XbaI
PstI
RB
ori ColE1
phaP
mf1
pha3'
pCGmf124
19.19 Kb
3'tml-npt-355
LB
Gm
ori pRI
KpnI
XbaI
BamHI
KpnI
EcoRI
XbaI
BamHI
PstI
RB
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ori ColE1
mf2
Pha3'
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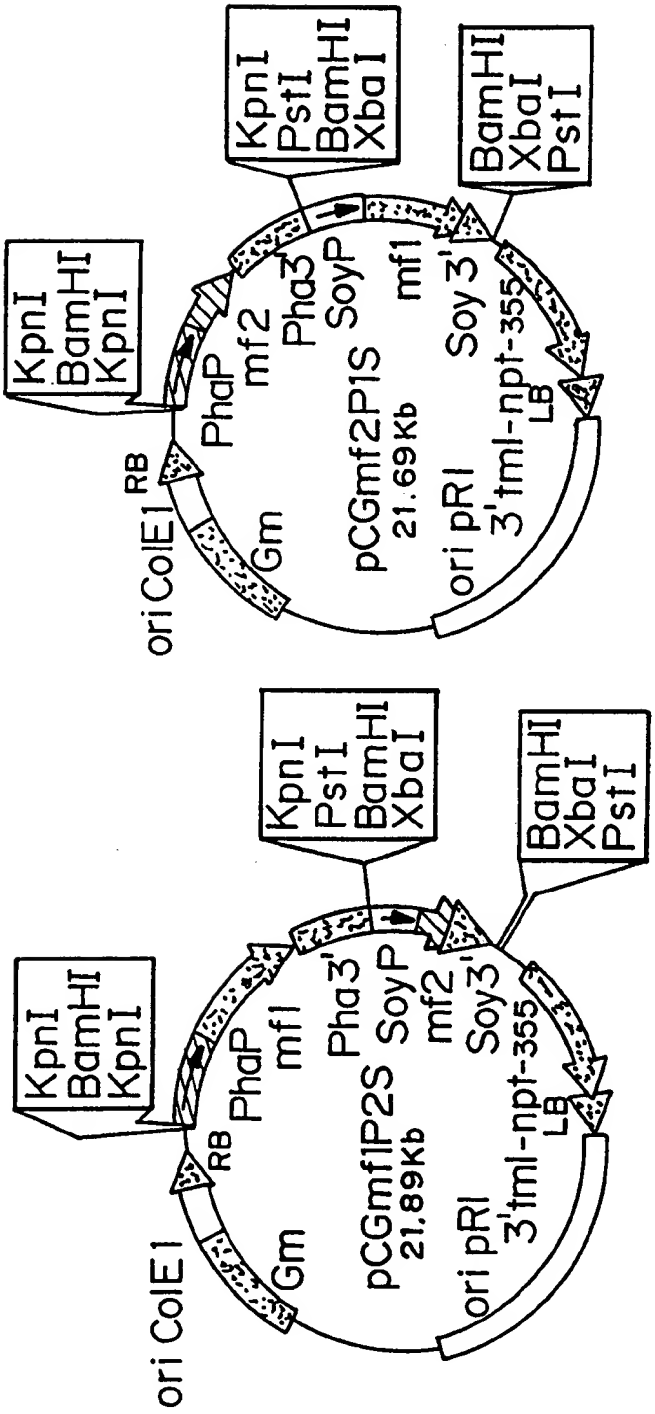
18.29 Kb
3'trI-npt-355
LB
Gm
ori pRI

FIG. 6B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PstI
BamHI
XbaI
BamHI
XbaI
PstI
KpnI
BamHI
KpnI
PhaP
mf2
Pha3'
SoyP
mf1
Soy3'
3'tml-npt-355
LB
RB
ori ColE1
Gm
pCGmf2P1S
21.69Kb
ori pRI
KpnI
PstI
BamHI
XbaI
BamHI
XbaI
PstI
KpnI
BamHI
KpnI
PhaP
mf1
Pha3'
SoyP
mf2
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<120> Modification of Fatty Acid Metabolism in Plants

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tctcagaaat ttggtagaat cattaacacc gcttcccctg ccggtctatt tggaaatttt 480
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gctgctggat tctttggaca gctcagatgg gagaggtctt ctggacaaat tttcaatcca 780
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taa 2703

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<213> Saccharomyces

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20 25 30

Gly Ala Lys Val Val Val Asn Asp Leu Gly Gly Thr Leu Gly Gly Ser
35 40 45

```
Gly His Asn Ser Lys Ala Ala Asp Leu Val Val Asp Glu Ile Lys Lys
     50                  55                  60

Ala Gly Gly Ile Ala Val Ala Asn Tyr Asp Ser Val Asn Glu Asn Gly
 65                  70                  75                  80

Glu Lys Ile Ile Glu Thr Ala Ile Lys Glu Phe Gly Arg Val Asp Val
                 85                  90                  95

Leu Ile Asn Asn Ala Gly Ile Leu Arg Asp Val Ser Phe Ala Lys Met
            100                 105                 110

Thr Glu Arg Glu Phe Ala Ser Val Val Asp Val His Leu Thr Gly Gly
        115                 120                 125

Tyr Lys Leu Ser Arg Ala Ala Trp Pro Tyr Met Arg Ser Gln Lys Phe
    130                 135                 140

Gly Arg Ile Ile Asn Thr Ala Ser Pro Ala Gly Leu Phe Gly Asn Phe
145                 150                 155                 160

Gly Gln Ala Asn Tyr Ser Ala Ala Lys Met Gly Leu Val Gly Leu Ala
                165                 170                 175

Glu Thr Leu Ala Lys Glu Gly Ala Lys Tyr Asn Ile Asn Val Asn Ser
            180                 185                 190

Ile Ala Pro Leu Ala Arg Ser Arg Met Thr Glu Asn Val Leu Pro Pro
        195                 200                 205

His Ile Leu Lys Gln Leu Gly Pro Glu Lys Ile Val Pro Leu Val Leu
    210                 215                 220

Tyr Leu Thr His Glu Ser Thr Lys Val Ser Asn Ser Ile Phe Glu Leu
225                 230                 235                 240

Ala Ala Gly Phe Phe Gly Gln Leu Arg Trp Glu Arg Ser Ser Gly Gln
                245                 250                 255

Ile Phe Asn Pro Asp Pro Lys Thr Tyr Thr Pro Glu Ala Ile Leu Asn
            260                 265                 270

Lys Trp Lys Glu Ile Thr Asp Tyr Arg Asp Lys Pro Phe Asn Lys Thr
        275                 280                 285

Gln His Pro Tyr Gln Leu Ser Asp Tyr Asn Asp Leu Ile Thr Lys Ala
    290                 295                 300
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Lys Lys Leu Pro Pro Asn Glu Gln Gly Ser Val Lys Ile Lys Ser Leu
305                 310                 315                 320

Cys Asn Lys Val Val Val Val Thr Gly Ala Gly Gly Gly Leu Gly Lys
                325                 330                 335

Ser His Ala Ile Trp Phe Ala Arg Tyr Gly Ala Lys Val Val Val Asn
            340                 345                 350

Asp Ile Lys Asp Pro Phe Ser Val Val Glu Glu Ile Asn Lys Leu Tyr
        355                 360                 365

Gly Glu Gly Thr Ala Ile Pro Asp Ser His Asp Val Val Thr Glu Ala
    370                 375                 380

Pro Leu Ile Ile Gln Thr Ala Ile Ser Lys Phe Gln Arg Val Asp Ile
385                 390                 395                 400

Leu Val Asn Asn Ala Gly Ile Leu Arg Asp Lys Ser Phe Leu Lys Met
                405                 410                 415

Lys Asp Glu Glu Trp Phe Ala Val Leu Lys Val His Leu Phe Ser Thr
            420                 425                 430

Phe Ser Leu Ser Lys Ala Val Trp Pro Ile Phe Thr Lys Gln Lys Ser
        435                 440                 445

Gly Phe Ile Ile Asn Thr Thr Ser Thr Ser Gly Ile Tyr Gly Asn Phe
    450                 455                 460

Gly Gln Ala Asn Tyr Ala Ala Ala Lys Ala Ala Ile Leu Gly Phe Ser
465                 470                 475                 480

Lys Thr Ile Ala Leu Glu Gly Ala Lys Arg Gly Ile Ile Val Asn Val
                485                 490                 495

Ile Ala Pro His Ala Glu Thr Ala Met Thr Lys Thr Ile Phe Ser Glu
            500                 505                 510

Lys Glu Leu Ser Asn His Phe Asp Ala Ser Gln Val Ser Pro Leu Val
        515                 520                 525

Val Leu Leu Ala Ser Glu Glu Leu Gln Lys Tyr Ser Gly Arg Arg Val
    530                 535                 540

Ile Gly Gln Leu Phe Glu Val Gly Gly Gly Trp Cys Gly Gln Thr Arg
545                 550                 555                 560
```

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Trp Gln Arg Ser Ser Gly Tyr Val Ser Ile Lys Glu Thr Ile Glu Pro
                565                 570                 575

Glu Glu Ile Lys Glu Asn Trp Asn His Ile Thr Asp Phe Ser Arg Asn
            580                 585                 590

Thr Ile Asn Pro Ser Ser Thr Glu Glu Ser Ser Met Ala Thr Leu Gln
        595                 600                 605

Ala Val Gln Lys Ala His Ser Ser Lys Glu Leu Asp Asp Gly Leu Phe
    610                 615                 620

Lys Tyr Thr Thr Lys Asp Cys Ile Leu Tyr Asn Leu Gly Leu Gly Cys
625                 630                 635                 640

Thr Ser Lys Glu Leu Lys Tyr Thr Tyr Glu Asn Asp Pro Asp Phe Gln
                645                 650                 655

Val Leu Pro Thr Phe Ala Val Ile Pro Phe Met Gln Ala Thr Ala Thr
            660                 665                 670

Leu Ala Met Asp Asn Leu Val Asp Asn Phe Asn Tyr Ala Met Leu Leu
        675                 680                 685

His Gly Glu Gln Tyr Phe Lys Leu Cys Thr Pro Thr Met Pro Ser Asn
    690                 695                 700

Gly Thr Leu Lys Thr Leu Ala Lys Pro Leu Gln Val Leu Asp Lys Asn
705                 710                 715                 720

Gly Lys Ala Ala Leu Val Val Gly Gly Phe Glu Thr Tyr Asp Ile Lys
                725                 730                 735

Thr Lys Lys Leu Ile Ala Tyr Asn Glu Gly Ser Phe Phe Ile Arg Gly
            740                 745                 750

Ala His Val Pro Pro Glu Lys Glu Val Arg Asp Gly Lys Arg Ala Lys
        755                 760                 765

Phe Ala Val Gln Asn Phe Glu Val Pro His Gly Lys Val Pro Asp Phe
    770                 775                 780

Glu Ala Glu Ile Ser Thr Asn Lys Asp Gln Ala Ala Leu Tyr Arg Leu
785                 790                 795                 800

Ser Gly Asp Phe Asn Pro Leu His Ile Asp Pro Thr Leu Ala Lys Ala
                805                 810                 815
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Val Lys Phe Pro Thr Pro Ile Leu His Gly Leu Cys Thr Leu Gly Ile
820 825 830

Ser Ala Lys Ala Leu Phe Glu His Tyr Gly Pro Tyr Glu Glu Leu Lys
835 840 845

Val Arg Phe Thr Asn Val Val Phe Pro Gly Asp Thr Leu Lys Val Lys
850 855 860

Ala Trp Lys Gln Gly Ser Val Val Val Phe Gln Thr Ile Asp Thr Thr
865 870 875 880

Arg Asn Val Ile Val Leu Asp Asn Ala Ala Val Lys Leu Ser Gln Ala
885 890 895

Lys Ser Lys Leu
900

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gatagttatg aacaagcctt gagaagagat gatgtgaagg caattgttgt tacaggtgca 180
aagggaaaat tttctggtgg ctttgatata actgcttttg gtgtactcca aggaggaaag 240
ggggagcaac caaatgttag aaacatatca attgaaatga tcactgatat ttttgaagct 300
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ttggggttag tggatgccat tgtccctccc gaagagttga tcaacactgc acgtagatgg 600
gctcttgaaa tcctagagcg gagaagacca tgggttcaca gtcttcacag gactgacaag 660
ttagagtctc ttgctgaggc taggaaaata tttaacttag ctagagctca ggcaaagaaa 720
cagtacccaa atcttaagca tacaattgcc tgcattgatg ctgttgaaac gggtgtcgtc 780
tctggccctc gtgctggact ttggaaggag gctgaagaat ttcagggact cctacattct 840
gatacttgca aaagcttaat tcatatcttc tttgcccagc gttcaacaac taaggtacct 900

ggagttactg atctgggttt ggtaccgaga caaatcaaga aagttgctat tgtcggagga 960
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aaagaagtga acgataagtt cttgcaggct ggcattgaca gagtcagagc aaacctacaa 1080
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20 25 30

Phe Asp Val Leu Phe Ser Leu Arg Asp Ser Tyr Glu Gln Ala Leu Arg
35 40 45

Arg Asp Asp Val Lys Ala Ile Val Val Thr Gly Ala Lys Gly Lys Phe
50 55 60

Ser Gly Gly Phe Asp Ile Thr Ala Phe Gly Val Leu Gln Gly Gly Lys
65 70 75 80

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Gly Glu Gln Pro Asn Val Arg Asn Ile Ser Ile Glu Met Ile Thr Asp
                 85                  90                  95

Ile Phe Glu Ala Ala Arg Lys Pro Ala Val Ala Ala Ile Asp Gly Leu
            100                 105                 110

Ala Leu Gly Gly Gly Leu Glu Val Ala Met Ala Cys His Ala Arg Ile
        115                 120                 125

Ser Thr Pro Thr Ala Gln Leu Gly Leu Pro Glu Leu Gln Leu Gly Ile
    130                 135                 140

Ile Pro Gly Phe Gly Gly Thr Gln Arg Leu Pro Arg Leu Val Gly Leu
145                 150                 155                 160

Ser Lys Ala Leu Glu Met Met Leu Thr Ser Lys Pro Ile Lys Gly Gln
                165                 170                 175

Glu Ala His Ser Leu Gly Leu Val Asp Ala Ile Val Pro Pro Glu Glu
            180                 185                 190

Leu Ile Asn Thr Ala Arg Arg Trp Ala Leu Glu Ile Leu Glu Arg Arg
        195                 200                 205

Arg Pro Trp Val His Ser Leu His Arg Thr Asp Lys Leu Glu Ser Leu
    210                 215                 220

Ala Glu Ala Arg Lys Ile Phe Asn Leu Ala Arg Ala Gln Ala Lys Lys
225                 230                 235                 240

Gln Tyr Pro Asn Leu Lys His Thr Ile Ala Cys Ile Asp Ala Val Glu
                245                 250                 255

Thr Gly Val Val Ser Gly Pro Arg Ala Gly Leu Trp Lys Glu Ala Glu
            260                 265                 270

Glu Phe Gln Gly Leu Leu His Ser Asp Thr Cys Lys Ser Leu Ile His
        275                 280                 285

Ile Phe Phe Ala Gln Arg Ser Thr Thr Lys Val Pro Gly Val Thr Asp
    290                 295                 300

Leu Gly Leu Val Pro Arg Gln Ile Lys Lys Val Ala Ile Val Gly Gly
305                 310                 315                 320

Gly Leu Met Gly Ser Gly Ile Ala Thr Ala Leu Ile Leu Ser Asn Tyr
                325                 330                 335
```

```
His Val Val Leu Lys Glu Val Asn Asp Lys Phe Leu Gln Ala Gly Ile
            340                 345                 350

Asp Arg Val Arg Ala Asn Leu Gln Ser Arg Val Lys Lys Gly Asn Met
        355                 360                 365

Thr Asn Glu Lys Phe Glu Lys Ser Ile Ser Leu Leu Lys Gly Val Leu
    370                 375                 380

Asn Tyr Glu Ser Phe Lys Asp Val Asp Met Val Ile Glu Ala Val Ile
385                 390                 395                 400

Glu Asn Val Ser Leu Lys Gln Gln Ile Phe Ser Asp Leu Glu Lys Tyr
                405                 410                 415

Cys Pro Pro His Cys Met Leu Ala Thr Asn Thr Ser Thr Ile Asp Leu
            420                 425                 430

Glu Leu Ile Gly Glu Arg Ile Lys Ser Arg Asp Arg Ile Ile Gly His
        435                 440                 445

Thr Ala Ala Gln Val Ile Val Asp Leu Leu Asp Val Gly Lys Asn Ile
    450                 455                 460

Lys Lys Thr Pro Val Val Val Gly Asn Cys Thr Gly Phe Ala Val Asn
465                 470                 475                 480

Arg Met Phe Phe Pro Tyr Ser Gln Ala Ala Ile Leu Leu Ala Glu His
                485                 490                 495

Gly Val Asp Pro Tyr Gln Ile Asp Arg Ala Ile Ser Lys Phe Gly Met
            500                 505                 510

Pro Met Gly Pro Phe Arg Leu Cys Asp Leu Val Gly Phe Gly Val Ala
        515                 520                 525

Ala Ala Thr Ala Ser Gln Phe Val Gln Ala Phe Pro Glu Arg Thr Tyr
    530                 535                 540

Lys Ser Met Leu Ile Pro Leu Met Gln Glu Asp Lys Asn Ala Gly Glu
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Ser Thr Arg Lys Gly Phe Tyr Val Tyr Asp Lys Asn Arg Lys Ala Gly
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Pro Asn Pro Glu Leu Lys Lys Tyr Ile Glu Lys Ala Arg Asn Ser Ser
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Gly Val Ser Val Asp Pro Lys Leu Thr Lys Leu Pro Glu Lys Asp Ile
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Val Glu Met Ile Phe Phe Pro Val Val Asn Glu Ala Cys Arg Val Leu
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Ala Glu Gly Ile Ala Val Lys Ala Ala Asp Leu Asp Ile Ala Gly Val
625 630 635 640

Met Gly Met Gly Phe Pro Ser Tyr Arg Gly Gly Leu Met Phe Trp Ala
645 650 655

Asp Ser Leu Gly Ser Asn Tyr Ile Tyr Ser Arg Leu Glu Glu Trp Ser
660 665 670

Lys Gln Tyr Gly Gly Phe Phe Lys Pro Cys Gly Tyr Leu Ala Glu Arg
675 680 685

Ala Val Gln Gly Ala Thr Leu Ser Ala Pro Gly Gly His Ala Lys Pro
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Ile Val Arg Thr Lys
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<210> 5
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<400> 5
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<210> 6
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:

oligonucleotide primer- Primer 2

<400> 6
caacccgaag gtgccgccat t 21

<210> 7
<211> 3907
<212> DNA
<213> Pseudomonas putida

<220>
<221> gene
<222> (1)..(3907)
<223> faoAB gene sequence

<400> 7
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cgtctga 3907

<210> 8
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer GVR471

<400> 8
cggtacccat tgtactccca gtatcat 27

<210> 9

<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer GVR472

<400> 9
catttaaata gtagagtatt gaatatg 27

<210> 10
<211> 1558
<212> DNA
<213> Bean Phaseolin

<220>
<221> promoter
<222> (1)..(1558)

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<210> 11
<211> 983
<212> DNA
<213> Soybean oleosin

<220>
<221> promoter
<222> (1)..(983)

<400> 11
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aacttcacct taaccattta atg 983

<210> 12
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer JA408

<400> 12
tctagataca tccatttctt aatataatcc tcttattc 38

<210> 13
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- np1

<400> 13
catttaaatg gttaaggtga aggtagggct 30

<210> 14
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer np2

<400> 14
aagcttaaaa tgatttacga aggtaaagcc 30

<210> 15
<211> 22
<212> DNA
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<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer np3

<400> 15
attgctttca gttgaagcgc tg 22

<210> 16
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer np4

<400> 16
aagcttaaaa tgagcctgaa tccaagagac 30

<210> 17
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer np5

<400> 17
aagctttcag acgcgttcga agacagtg 28

<210> 18
<211> 56
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer N-fox2b

<400> 18
tcccccggga ggaggttttt attatgcctg gaaatttatc cttcaaagat agagtt 56

<210> 19
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer N-bamfox2b

<400> 19
aaggatcctt gatgtcattt acaactacc 29

<210> 20
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer C-fox2

<400> 20
gctctagata gggaaagatg tatgtaag 28

<210> 21
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer C-bamfox2

<400> 21
tgacatcaag gatcctttt 19

<210> 22
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer GVR396

<400> 22
gatttaaatg caagcttaaa taagtatgaa ctaaaatgc 39

<210> 23
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
oligonucleotide primer- Primer GVR397

<400> 23
cggtacctta gttggtaggg tgcta 25

<210> 24
<211> 2162
<212> DNA
<213> Pseudomonas putida

<220>
<221> gene
<222> (1)..(2162)
<223> FaoA gene sequence

<400> 24
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tt 2162

<210> 25
<211> 1190
<212> DNA
<213> Pseudomonas putida

<220>
<221> gene
<222> (1)..(1190)
<223> FaoB gene sequence

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<210> 26
<211> 391
<212> PRT
<213> Pseudomonas putida

<220>
<221> PEPTIDE
<222> (1)..(391)
<223> FaoB amino acid sequence

<400> 26
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Pro Met Gly Arg Ser Lys Gly Gly Met His Arg Asn Thr Arg Ala Glu
20 25 30

Asp Met Ser Ala His Leu Ile Ser Lys Leu Leu Glu Arg Asn Gly Lys
35 40 45

Val Asp Pro Lys Glu Val Glu Asp Val Ile Trp Gly Cys Val Asn Gln
50 55 60

Thr Leu Glu Gln Gly Trp Asn Ile Ala Arg Met Ala Ser Leu Met Thr
65 70 75 80

Pro Ile Pro His Thr Ser Ala Ala Gln Thr Val Ser Arg Leu Cys Gly
85 90 95

Ser Ser Met Ser Ala Leu His Thr Ala Ala Gln Ala Ile Met Thr Gly
100 105 110

Asn Gly Asp Val Phe Val Val Gly Gly Val Glu His Met Gly His Val
115 120 125

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Ser Met Met His Gly Val Asp Pro Asn Pro His Leu Ser Leu His Ala
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Ala Lys Ala Ser Gly Met Met Gly Leu Thr Ala Glu Met Leu Gly Lys
145                 150                 155                 160

Met His Gly Ile Thr Arg Glu Gln Gln Asp Leu Phe Gly Leu Arg Ser
                165                 170                 175

His Gln Leu Ala His Lys Ala Thr Val Glu Gly Lys Phe Lys Asp Glu
            180                 185                 190

Ile Ile Pro Met Gln Gly Tyr Asp Glu Asn Gly Phe Leu Lys Val Phe
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Asp Phe Asp Glu Thr Ile Arg Pro Glu Thr Thr Leu Glu Gly Leu Ala
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Ser Leu Lys Pro Ala Phe Asn Pro Lys Gly Gly Thr Val Thr Ala Gly
225                 230                 235                 240

Thr Ser Ser Gln Ile Thr Asp Gly Ala Ser Cys Met Ile Val Met Ser
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Gly Gln Arg Ala Met Asp Leu Gly Ile Gln Pro Leu Ala Val Ile Arg
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Ser Met Ala Val Ala Gly Val Asp Pro Ala Ile Met Gly Tyr Gly Pro
        275                 280                 285

Val Pro Ser Thr Gln Lys Ala Leu Lys Arg Ala Gly Leu Thr Met Ala
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Asp Ile Asp Phe Ile Glu Leu Asn Glu Ala Phe Ala Ala Gln Ala Leu
305                 310                 315                 320

Pro Val Leu Lys Asp Leu Lys Val Leu Asp Lys Met Asp Glu Lys Val
                325                 330                 335

Asn Leu His Gly Gly Ala Ile Ala Leu Gly His Pro Phe Gly Cys Ser
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Gly Ala Arg Ile Ser Gly Thr Leu Leu Asn Val Met Lys Gln Asn Gly
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<213> Artificial Sequence

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35 40 45

Ala Asp Ala Ser Val Lys Gly Val Ile Val Arg Ser Gly Lys Asp Val

50 55 60

Phe Ile Val Gly Ala Asp Ile Thr Glu Phe Val Asp Asn Ph Lys Leu
65 70 75 80

Pro Glu Ala Glu Leu Val Ala Gly Asn Leu Glu Ala Asn Arg Ile Phe
85 90 95

Asn Ala Phe Glu Asp Leu Glu Val Pro Thr Val Ala Ala Ile Asn Gly
100 105 110

Ile Ala Leu Gly Gly Gly Leu Glu Met Cys Leu Ala Ala Asp Tyr Arg
115 120 125

Val Met Ser Thr Ser Ala Arg Ile Gly Leu Pro Glu Val Lys Leu Gly
130 135 140

Ile Tyr Pro Gly Phe Gly Gly Thr Val Arg Leu Pro Arg Leu Ile Gly
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Ser Asp Asn Ala Ile Glu Trp Ile Ala Ala Gly Lys Glu Asn Arg Ala
165 170 175

Glu Asp Ala Leu Lys Val Gly Ala Val Asp Ala Val Val Ala Pro Glu
180 185 190

Leu Leu Leu Ala Gly Ala Leu Asp Leu Ile Lys Arg Ala Ile Ser Gly
195 200 205

Glu Leu Asp Tyr Lys Ala Lys Arg Gln Pro Lys Leu Glu Lys Leu Lys
210 215 220

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225 230 235 240

Val Ala Gly Gln Ala Gly Pro Asn Tyr Pro Ala Pro Val Glu Ala Ile
245 250 255

Lys Ser Ile Gln Lys Ala Ala Asn Phe Gly Arg Asp Lys Ala Leu Glu
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Val Glu Ala Ala Gly Phe Ala Lys Leu Ala Lys Thr Ser Val Ala Glu
275 280 285

Ser Leu Ile Gly Leu Phe Leu Asn Asp Gln Glu Leu Lys Arg Lys Ala
290 295 300

Lys Ala His Asp Glu Ile Ala His Asp Val Lys Gln Ala Ala Val Leu

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325 330 335

Gly Thr Pro Ile Leu Met Lys Asp Ile Arg Glu Glu Ala Ile Gln Leu
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Arg Leu Thr Pro Ala Lys Met Ala Glu Ala Leu Asn Ala Ile Arg Pro
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Thr Leu Ser Tyr Gly Asp Phe Ala Asn Val Asp Ile Val Val Glu Ala
385 390 395 400

Val Val Glu Asn Pro Lys Val Lys Gln Ala Val Leu Ala Glu Val Glu
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Gly Gln Val Lys Asp Asp Ala Ile Leu Ala Ser Asn Thr Ser Thr Ile
420 425 430

Ser Ile Asn Leu Leu Ala Lys Ala Leu Lys Arg Pro Glu Asn Phe Val
435 440 445

Gly Met His Phe Phe Asn Pro Val His Met Met Pro Leu Val Glu Val
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Ile Arg Gly Glu Lys Ser Ser Asp Val Ala Val Ala Thr Thr Val Ala
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Tyr Ala Lys Lys Met Gly Lys Asn Pro Ile Val Val Asn Asp Cys Pro
485 490 495

Gly Phe Leu Val Asn Arg Val Leu Phe Pro Tyr Phe Gly Gly Phe Ala
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Lys Leu Val Ser Ala Gly Val Asp Phe Val Arg Ile Asp Lys Val Met
515 520 525

Glu Lys Phe Gly Trp Pro Met Gly Pro Ala Tyr Leu Met Asp Val Val
530 535 540

Gly Ile Asp Thr Gly His His Gly Arg Asp Val Met Ala Glu Gly Phe
545 550 555 560

Pro Asp Arg Met Lys Asp Glu Arg Arg Ser Ala Val Asp Ala Leu Tyr

565 570 575

Glu Ala Asn Arg Leu Gly Gln Lys Asn Gly Lys Gly Phe Tyr Ala Tyr
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Glu Thr Asp Lys Arg Gly Lys Pro Lys Lys Val Phe Asp Ala Thr Val
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Leu Asp Val Leu Lys Pro Ile Val Phe Glu Gln Arg Glu Val Thr Asp
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Glu Asp Ile Ile Asn Trp Met Met Val Pro Leu Cys Leu Glu Thr Val
625 630 635 640

Arg Cys Leu Glu Asp Gly Ile Val Glu Thr Ala Ala Glu Ala Asp Met
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Gly Leu Val Tyr Gly Ile Gly Phe Pro Pro Phe Arg Gly Gly Ala Leu
660 665 670

Arg Tyr Ile Asp Ser Ile Gly Val Ala Glu Phe Val Ala Leu Ala Asp
675 680 685

Gln Tyr Ala Asp Leu Gly Pro Leu Tyr His Pro Thr Ala Lys Leu Arg
690 695 700

Glu Met Ala Lys Asn Gly Gln Arg Phe Phe Asn
705 710 715

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 99/04999

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DENG W L ET AL: "EXPRESSION OF SOYBEAN-EMBRYO LIPOXYGENASE-2 IN TRANSGENIC TOBACCO TISSUE" PLANTA, (MAY 1992) VOL. 187, NO. 2, PP. 203-208. ISSN: 0032-0935., XP002108604 UNIV KENTUCKY, DEPT AGRON, LEXINGTON, KY, 40546;UNIV KENTUCKY, DEPT HORT & LANDSCAPE ARCHITECTURE, LEXINGTON, KY, 40546 the whole document ---	1,6,9, 10,13, 18-23
X	WO 98 00557 A (MONSANTO CO) 8 January 1998 (1998-01-08) see esp. pp.5-128; ex.10 --- -/--	1-3, 5-15, 17-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1999	21/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	OLESEN C ET AL: "The glyoxysomal 3-ketoacyl-CoA thiolase precursor from Brassica napus has enzymatic activity when synthesized in Escherichia coli." FEBS LETTERS, (1997 JUL 21) 412 (1) 138-40. , XP002108605 the whole document	1-24
A	ISHIKAWA M. ET AL.: "Reconstitution, morphology and crystallization of a fatty acid beta-oxidation multienzyme complex from Pseudomonas fragi" BIOCHEMICAL JOURNAL, vol. 328, 1997, pages 815-820, XP002108606 the whole document	1-24
A	HUIJBERTS G N M ET AL: "13C NUCLEAT MAGNETIC RESONANCE STUDIES OF PSEUDONOMAS PUTIDA FATTY ACID METABOLIC ROUTS INVOLVED IN POLY(3-HYDROXYALKANOATE) SYNTHESIS" JOURNAL OF BACTERIOLOGY, vol. 176, no. 6, 1 March 1994 (1994-03-01), pages 1661-1666, XP002045277 ISSN: 0021-9193 the whole document	1-24
A	WO 98 06854 A (MONSANTO CO) 19 February 1998 (1998-02-19) see esp. ex.10	1-24
A	WO 94 23027 A (ZENECA LTD ;SLABAS ANTHONY RYSARD (GB); ELBOROUGH KEIRAN MICHAEL ( ) 13 October 1994 (1994-10-13) the whole document	1-24
A	LEIJ VAN DER F R ET AL: "STRATEGIES FOR THE SUSTAINABLE PRODUCTION OF NEW BIODEGRADABLE POLYESTERS IN PLANTS: A REVIEW" CANADIAN JOURNAL OF MICROBIOLOGY, vol. 41, no. SUPPL. 01, 1 January 1995 (1995-01-01), pages 222-238, XP002045278 ISSN: 0008-4166 cited in the application see esp. p.228/29; p.233 1.col. 1.par.	1-24
A	WO 98 06831 A (MAX PLANCK GESELLSCHAFT ;WILLMITZER LOTHAR (DE); COGNATA URSULA (D) 19 February 1998 (1998-02-19) see esp. p.7 1.par.; p.17 2.par.; claims	1-24

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 99/04999

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 10703 A (UNIV WASHINGTON) 27 March 1997 (1997-03-27) see esp. p.228/29; p.233 1.col. 1.par.	24
A	DE 195 31 678 A (SHERIFF AHMED DR) 6 March 1997 (1997-03-06) the whole document	24
T	EP 0 894 864 A (BRENNICKE AXEL DR ;BINDER STEFAN DR (DE)) 3 February 1999 (1999-02-03) the whole document	1-24

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intr ional Application No
PCT/US 99/04999

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 2259251 A	08-01-1998
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		AU 3982897 A	06-03-1998
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		CA 2232623 A	27-03-1997
		CN 1196655 A	21-10-1998
		EP 0868119 A	07-10-1998
		NZ 320845 A	28-10-1998
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EP 0894864 A	03-02-1999	NONE	